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Hybrid Paneer (cottage cheese): Influence of mung bean protein isolate on the texture, microstructure, and gastro-small intestinal digestion *in vitro* of paneer

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Dedication

I thank almighty God.

My father Tojan Thomas, mother Shiny Tojan, and my brother Shaun Tojan for their constant love and support. I thank all the people in my life who have helped me to become the person who I am today.

Abstract

The global population is estimated to grow to about 9.6 billion by 2050 thus raising the demand for animal-based protein. Due to the huge rise in population, there is a rising concern among the industries as well as government agencies regarding feeding the growing population. Partially replacing milk proteins with plant protein isolate is an interesting opportunity to develop novel food products with unique functionalities along with reducing the demand for animal proteins. Mung bean protein due to its high protein content, health benefits, and good functional properties can be utilised as a potential source of high-quality protein ingredients.

The effect of partially replacing cow milk with mung bean protein isolate (MBPI) on the texture, microstructure and digestibility of paneer (acid-heat coagulated cheese) was investigated in this study in order to identify the potential of MBPI to partially replace milk proteins. Cow milk-mung bean paneer (CMMBP) was successfully developed by replacing 30 % of the milk with MBPI by keeping the overall protein content similar to cow milk. The developed CMMBP had higher protein and moisture content when compared to cow milk paneer (CMP) and was shown to have lower fat content. Colour analysis showed a slightly darker colour in CMMBP with the incorporation of MBPI.

Small amplitude oscillatory tests showed higher storage modulus (G') values than loss modulus (G'') values throughout the frequency range and showed a higher G' value in CMMBP compared to CMP, indicating greater elastic properties. Loss tangent ($Tan \delta$) values of both the paneer samples were always less than one indicating a dominant solid-like nature. Texture profile analysis of the samples showed a significant reduction in the textural properties like hardness, cohesiveness, chewiness, and springiness values in CMMBP ($P < 0.05$). This may be due to the presence of globular mung bean proteins entrapped within the milk protein network thus preventing the formation of a compact protein network resulting in lower textural attributes. This is supported by the confocal microscopy results that showed protein aggregates of varying sizes within the casein matrix in CMMBP in contrast to a much uniform structure in CMP.

In vitro gastro small intestinal digestion of the paneer samples was also performed according to INFOGEST protocol to evaluate its protein digestibility under stimulated gastric phase for 1 hour and stimulated small intestinal phase for 2 hr. The digests were analysed using SDS-PAGE and ninhydrin assay for determining the protein hydrolysis and free amino N values during digestion. Ninhydrin results showed a lower release of free amino N in the gastric phase

compared to the small intestinal phase in both samples. The free amino N release in CMMBP was significantly lower than CMP during the small intestinal phase ($P < 0.05$). This can be further confirmed by the results of SDS-PAGE which showed the presence of resistant peptides during the intestinal phase in CMMBP, depicting a lower rate of protein digestion. This can be explained due to the presence of anti-nutritional factors, the structural arrangement of proteins and complex formation due to the interaction of proteins with other seed components present in MBPI.

Further studies on the addition of calcium chloride and hydrocolloids like carboxymethyl cellulose and pre-gelatinised starch need to be done in relation to the improvement of textural and sensory properties of paneer. The effect of various processing techniques on the digestibility of plant protein added to the protein formulation also needs to be studied.

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List of Abbreviations

MF	Milk fat
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CMP	Cow Milk Paneer
CMMBP	Cow Milk-Mung Bean Paneer
BSA	Blood serum albumin
CCP	Colloidal calcium phosphate
GHG	Greenhouse gas
FAO	Food and Agriculture Organization
MBP	Mung bean protein
MBPI	Mung bean protein isolate
GIT	Gastrointestinal tract
GDL	Glucono-delta lactone
LDL	Low density lipoprotein
WHO	World Health Organization
LGC	Least gelation concentration
CLSM	Confocal laser scanning microscopy
TPA	Texture profile analysis

Chapter 1 Introduction

Paneer is a type of fresh, soft, unripened cheese made by the acid-heat coagulation of milk. It is quite popular in the Indian sub-continent and is used in the preparation of various culinary dishes and snacks. Paneer is highly nutritious and is rich in proteins, fats, vitamins, and minerals like calcium and phosphorous (Khan & Pal, 2011; Kumar et al., 2014). The global paneer market reached 1.9 million tons by 2020 and shows an increasing future market trend. Growing population, changing dietary habits, exploration of inter-cultural foods and introduction of paneer in western fast foods are some of the major factors behind the increasing global demand for paneer (IMARC, 2021).

Despite the growing demand, there is a gradual decline in the use of dairy-based products due to changing customer lifestyle and health concerns associated with the consumption of milk products, such as various allergic reactions in certain individuals, antibiotic residues in milk, and cholesterol. The decline is also because of increasing environmental concerns and consumer awareness regarding the negative impacts of the dairy industry on the environment (Jeske et al., 2018). However, due to the rise in population, there is a huge demand for proteins which has left industries looking for more sustainable and innovative alternative protein ingredients (Boland et al., 2013).

The utilisation of more affordable, accessible, and nutritious substitutes like legume proteins can be an effective way of overcoming the global protein challenge. Legume proteins are one of the finest sources of protein and other essential nutrients (Cheng et al., 2019). Legume seeds have a protein content of 20-25 % which is 2-3 times greater than cereals (Cai et al., 2001). Other than their protein content, legume proteins are also known for their bioactive peptides, partially digested proteins of various lengths, and even intact proteins, that can provide numerous physiological and positive health benefits, which include improved cardiovascular health, lower cancer risk, lower inflammation, weight control and improved insulin sensitivity (Carbonaro et al., 2015).

Mung bean belongs to the family of legumes and has a protein content of 20-27 %. It is rich in nutrients and has a high concentration of calcium, iron, manganese, along with vitamin B and C. It also has a good protein digestibility compared to proteins of other legumes (Fan & Sosulski, 1974). Mung bean protein has a similar amino acid profile to soybean protein and matches well with FAO/WHO reference protein (Brishti et al., 2017; Fan & Sosulski, 1974;

Thompson, 1977). The 8S globulin of mung bean protein isolate (MBPI) is structurally similar to β -conglycinin present in soybean (Hou et al., 2019). The similarity between these two protein components can have similar functionalities and applications (Cai et al., 2001). The allergenicity of mung bean is comparatively lower and was found to decrease in the order of peanut > soybean > lentil > chickpea > pea > mung bean (Verma et al., 2013). Several researchers have also reported the highest yield of bean curd produced using mung bean protein when compared to other legume proteins (Cai et al., 2001; Cai et al., 2002).

The gelling ability of milk proteins has been greatly utilised by industries for the manufacturing of dairy products (Lucey, 2020). Similarly, plant proteins show the ability to form gels or curds by forming a matrix to hold water, flavours and other nutrients. For example, tofu made from soybean curd is a food of cultural significance since ancient times in Asia and has been gaining popularity in western society (Cai & Baik, 2001). The gelling ability of mung bean protein was found to be better than soy protein (Brishti et al., 2017). Therefore, this property can be utilised favourably for partially replacing milk proteins with plant proteins.

Due to all these favourable characteristics, mung bean has a huge potential to be used in different value-added products, however, except for only a few attempts made to produce mung bean curd, no other studies were found in literature about value-added products like paneer or other products made of mung bean. Partial replacement of dairy products with legume proteins is of high importance as it provides an opportunity to develop hybrid dairy-legume cheese analogues, that can open the door for new markets and for the development of innovative food products with low environmental impact along with improved nutritional and functional properties.

Recently, Ouyang et al. (2021) investigated the potential of plant proteins to be used as an ingredient to develop cheese with characteristic sensory properties tailored towards Southeast and East Asian customers. They also suggested the use of proteins common to the Southeast and East Asian population as it may be easier for them to accept familiar flavours.

The overall quality of a product cannot only be determined by its protein content alone, but also through other factors like digestibility and bioavailability (Lynch et al., 2018). To this date, no study has reported on the digestibility of paneer. This study can help to understand the breakdown and release of protein within the gastrointestinal tract (GIT). Another major question to be answered is the digestibility of plant and animal protein mixed systems. Knowledge of the difference between the kinetics of protein digestion in a single system and a

mixed system is essential for the manufacturing of products with high nutritional and biological value (Alves & Tavares, 2019).

Therefore, the present study was an attempt to develop a hybrid dairy-legume paneer and to characterise it in terms of textural, microstructural properties, and to study its gastro-small intestinal protein digestion *in vitro*.

This study had been conducted through the following objectives.

Objectives

- 1) To study the potential of developing a product similar to paneer by substituting cow milk with mung bean protein isolate at 30 % level.
- 2) To study the textural, rheological, and microstructural properties of 100 % cow milk paneer (CMP) and paneer made from cow milk-mung bean protein combination (CMMBP) and to understand the effects of mung bean protein isolate substitution on these properties.
- 3) To perform the protein digestion of CMP and CMMBP using an *in vitro* gastro-small intestinal model and to understand their protein digestion kinetics (0, 10, 30, 60, 70, 120, and 180 min) during gastro-small intestinal digestion.

Chapter 2 Literature Review

2.1 Paneer

2.1.1 Introduction

Paneer is a type of soft-unripened cheese, made by the coagulation of milk, using heat and acid. It is usually made using buffalo or cow milk and its properties greatly depend upon the composition of milk used. Buffalo milk is widely used over cow milk as it has a higher quantity of fat, casein, and minerals like calcium and phosphorous (Kumar et al., 2014). The milk is heated to 80-90°C and is cooled down to 70°C before coagulating using acids. Organic acids like citric acid, acetic acid, and ascorbic acid are mainly used for the manufacturing of paneer (Khan & Pal, 2011).

Paneer has a soft body that is tightly linked and has a smooth texture. It is known for its marble white colour and mild sweet acid-nutty taste (Khan & Pal, 2011). It is similar to Queso-Blanca, a type of cheese popular in South America, that is prepared in a manner similar to paneer (Farkye, 2017). Paneer is usually consumed fresh and has a low shelf life due to its high moisture content (Murtaza et al., 2017). It is used for the preparation of several culinary dishes and snacks in the Asian sub-continent, mainly India, especially due to its ability to retain shape during frying (Khan & Pal, 2011). Paneer is a highly nutritious and wholesome product that adds value to the diet, as it is rich in proteins, fat, vitamins, and minerals like calcium and phosphorous (Karadbhajne & Bhoyarkar, 2010). Therefore, paneer is considered as an optimal food for infants, children, pregnant women, and ageing people (Kumar et al., 2014). It is also a preferred source of protein for vegetarians. Frozen paneer is also available these days to address the issue of low shelf life. Due to its broad culinary applications and nutritional profile paneer has a huge potential to be popularised around the world (Agrawal & Sinha, 2018).

2.1.2 Composition of paneer

Paneer contains about 53-55 % moisture, 17-18 % protein, 23-26 % fat, 2-2.5 % lactose and 1.5-2 % minerals (Chandan, 2007). Paneer structure is a mixture of milk fat and other milk constituents entrapped within a network formed by casein micelles (Ahmed & Bajwa, 2019). The composition of paneer greatly depends upon the composition of milk used, the manufacturing method applied, and the quantity of milk solids lost in the whey (Kumar et al., 2014). Composition of paneer from previous studies are given in Table 1.

Table 1: Chemical composition of paneer manufactured using cow and buffalo milk having different concentrations of fat

Type of milk	Constituents (%)						Reference
	Moisture	Fat	Protein	Lactose	Ash	Total solids	
Buffalo milk (3.5 % fat)	56.99	18.10	18.43	-	-	-	(Chawla et al., 1987)
Buffalo milk (4 % fat)	54.05	23.27	16.78	2.69	2.20	-	(Chawla et al., 1987)
Buffalo milk (5 % fat)	56.77	22.30	-	-	-	-	(Bhattacharya et al., 1971)
Buffalo milk (6 % fat)	48.2	24.20	20.20	2.54	1.25	-	(Dongare & Syed, 2018)
Buffalo milk (7.1 % fat)	-	28.86	16.07	2.33	2.67	49.93	(Masud et al., 1992)
Buffalo milk (6 % fat)	50.4	23.67	18.75	-	2.17	49.6	(Masud et al., 2007)
Buffalo milk (6 % fat)	51.74	27.19	15.32	2.59	2.44	48.20	(Kumar et al., 2008)
Cow milk (3.5 % fat)	55.97	18.98	20.93	2.01	1.45	-	(Mistry et al., 1992)
Cow milk (4.5 % fat)	55.26	24.15	18.43	-	-	-	(Syed et al., 1992)
Cow milk (5 % fat)	53.90	24.80	17.60	-	-	-	(Singh & Kanawjia, 1988)
Cow milk (4.2 % fat)	-	22.46	21.23	2.52	2.53	48.74	(Masud et al., 1992)
Cow milk (2.65 % fat)	52.68	21.63	-	-	1.23	47.32	(Shanaziya et al., 2018)
Standardised milk	58.31	20.98	17.39	-	1.30	-	(Ahmed & Bajwa, 2019)
Goat milk (4.86 % fat)	46.94	26.95	19.99	-	1.93	53.06	(Agnihotri & Pal, 1996)
Cow milk	56.9	19.2	18.3	-	1.9	-	(Khan et al., 2014)
Cow milk and soy milk (50:50)	64.33	15.42	17.29	-	1.41	37.21	(Jadhavar et al., 2009)

2.1.3 Manufacturing process of paneer

There are several methods available for the manufacturing of paneer at different scales with slight variations found between processing steps and parameters. A standard method for the manufacturing of paneer on a pilot scale was developed by Bhattacharya et al. (1971) (Figure 1). In this method, buffalo milk having a fat content of 6 % is heated to 82°C on a cheese vat for a period of 5 min and is cooled to about 70°C. The milk is then coagulated using 1 % citric acid solution, which is slowly added to the milk, with continuous stirring until the whey is separated from the curd. The mixture is then left undisturbed for a period of 10 min making sure the temperature doesn't fall below 63°C. The whey is then separated from the curd using a muslin cloth. The curd is then collected in a hoop; a rectangular frame with an opening on both top and bottom (35×28×10 cm), using a muslin cloth. A wooden plank is kept on top of the hoop with about 45 kg of weight for about 15-20 min for pressing. The pressed blocks of curd are then removed and cut into pieces and dipped in cold water (4-6°C) for 2-3 hr. The pieces of paneer are then kept on a wooden surface to remove the excess water. Later the pieces are packed using parchment paper and is stored at $4 \pm 1^\circ\text{C}$.

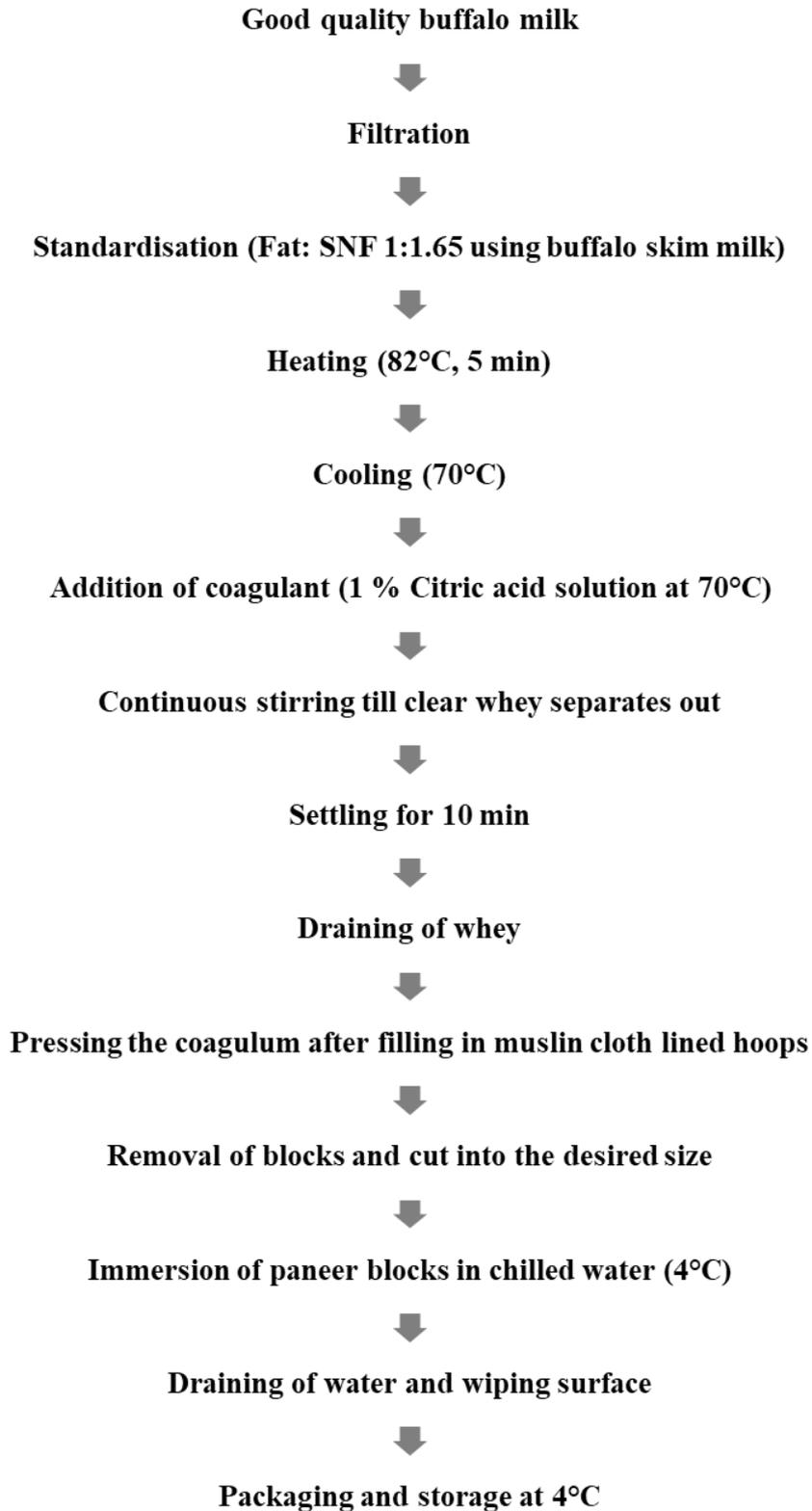


Figure 1: Flow diagram for manufacturing of paneer on a pilot-scale developed by Bhattacharya et al. (1971)
(Source: Kumar et al., 2014)

2.1.4 Factors influencing the quality of paneer

2.1.4.1 Type and quality of milk used

Different kinds of milk have been used for the manufacturing of paneer. Buffalo milk is generally used for the preparation of paneer of superior quality (Bhattacharya et al., 1971). Buffalo milk has varying physico-chemical properties when compared to cow milk such as the greater size of casein micelles, milk fat globules, and higher concentration of minerals like calcium and phosphorous. Casein micelles in buffalo milk have lower voluminosity and salvation properties compared to cow milk, which gives the paneer its superior quality and spongy texture (Sindhu, 1996).

Fat plays a major role in providing the paneer with superior taste and quality (Kumar et al., 2014). Previous studies on paneer by Ramasamy et al. (1999) and Masud (2002) recommended the use of buffalo milk with 6 % of fat for good quality paneer. This is supported by the studies of Bhattacharya et al. (1971) and Sachdeva and Singh (1988b) where they have used the buffalo milk standardised to 5-6 % for the preparation of paneer.

With several modifications to the manufacturing procedure, or by using additives, good quality paneer has also been developed using cow milk (Khan & Pal, 2011). Vishweshwaraiah and Anantkrishnan (1986) demonstrated that paneer prepared from cow milk containing 4.5 % fat has satisfied the Food safety and standards regulations, 2011 (FSSR). Although good quality paneer can be prepared from cow milk, it has lower sensory characteristics when compared to buffalo milk (Sindhu, 1996). Cow milk with 3.5 % fat has also been used to prepared paneer of satisfactory quality (Vishweshwaraiah & Anantkrishnan, 1986).

Due to seasonal variations and low availability of buffalo milk, cow milk is also mixed with buffalo milk to prepare paneer and has been found superior to the one produced with cow milk alone. Different proportions of buffalo and cow milk with different fat compositions were recommended by several researchers for the manufacturing of paneer (Khan & Pal, 2011). For example, good quality paneer has been successfully prepared using mixed milks, containing cow and buffalo milk in 1:1 ratio, with 5 % fat (Shukla, 1984).

According to the findings of Vishweshwaraiah and Anantkrishnan (1985) homogenisation of milk enhances the organoleptic qualities and yield of cow milk paneer. They have also found that highly acidic milk is not suitable for the production of paneer. De et al. (1971) found that milk with a titratable acidity between 0.20 - 0.23 % produced paneer of low quality, and the

milk with a titratable acidity higher than 0.28 % is not suitable for the manufacturing of paneer as it cannot be considered of acceptable quality due to the off-taste.

2.1.4.2 Heat treatment of milk

Heat treatment of milk is essential for the manufacturing of paneer. It not only kills the harmful microbes but also has a major influence on the sensory and physico-chemical properties of paneer. It helps to retain the total solids in milk thus increasing the yield of curd. Heating causes denaturing of whey proteins along with reducing the solubility of calcium phosphate which causes the whey proteins to attach itself to the surface of the casein and co-precipitate when treated with acid (Khan & Pal, 2011; Walstra & Jenness, 1984). Various time-temperature combinations were used by different researchers and are given in Table 2.

Table 2: Time-temperature combinations used by various researchers prior to acid coagulation (Khan & Pal, 2011)

Type of milk	Temperature-time combinations	Reference
Standardised buffalo milk (6 % fat)	82 °C/ 5 min	(Kumar et al., 2008)
Crossbred cow milk	85 °C/ no hold	(Bajwa et al., 2005)
Standardised buffalo milk (6 % fat)	85 °C/ 5 min	(Masud et al., 2007)
Skim milk	96 °C/ no hold	(Mendiratta et al., 2007)
Buffalo milk	95 °C/ 10 min	(Chawla et al., 1985)

2.1.4.3 Temperature of coagulation

Various parameters like body, texture, recovery of total solids, and yield are highly influenced by the coagulation temperature. Various researchers have adopted coagulation temperatures ranging from 60°C to 90°C and observed that moisture content decreased from 59 % to 49 % (Khan & Pal, 2011). Bhattacharya et al. (1971) and Sachdeva and Singh (1988b) recommend 70°C as the coagulation temperature for buffalo milk. Higher temperature produces paneer with hard and dry texture while lower coagulation temperature produces paneer with a wet surface (Sachdeva & Singh, 1988b). Many studies recommend a coagulation temperature of 80 - 85°C for the manufacturing of good quality paneer using cow milk (Sharma et al., 2002; Vishweshwariaiah & Anantakrishnan, 1985).

2.1.4.4 Type and concentration of acid used as the coagulant

The manufacturing of paneer involves acidifying the milk using various acids which results in the aggregation of milk proteins to form curds. Curds are basically proteins with fat and other solids entrapped within the protein network. The addition of acid reduces the pH of the milk to 4.6 which is the isoelectric point of casein, at this point the net charge becomes zero and causes the protein to form clumps thus forming curds. The yield, moisture content, body, and texture of paneer is greatly modified by the type and concentration of acid used (Khan & Pal, 2011). Different types of coagulating agents have been used in the past such as lactic acid (Kumar et al., 2008), ascorbic acid, citric acid (Sachdeva et al., 1985; Vishweshwariaiah & Anantakrishnan, 1985), malic acid, phosphoric acid, hydrochloric acid, cultured whey, yoghurt, etc (Khan & Pal, 2011). Citric acid at 1 % concentration is used commonly for the manufacturing of good quality paneer (Singh & Kanawjia, 1988) while some researchers like Vishweshwariaiah and Anantakrishnan (1985) suggested the use of 2 % citric acid for cow milk.

The addition of high concentrations of acids results in paneer with a hard body and low concentrations of acid result in paneer with a smooth texture and soft body. The quantity of coagulant required for coagulation depends on the nature of coagulant, temperature of coagulation, type of milk used, and its buffering capacity (Khan & Pal, 2011).

2.1.4.5 pH of coagulation

Coagulation pH plays a crucial role in determining the yield, texture, quality, and flavour of paneer (Khan & Pal, 2011). According to De (1980), with the reduction in the coagulation pH, the moisture content and yield decreased. According to Vishweshwariaiah and Anantakrishnan (1985), paneer prepared using cow milk had the best sensory properties at the coagulation pH of 5 and was superior to the one made at coagulation pH of 5.5. However, paneer coagulated at pH 5 had a lower yield, moisture, and total solids recovery. A coagulation pH of 5.20 - 5.25 was suggested by Sachdeva et al. (1991) for cow milk paneer.

2.1.4.6 Drainage of whey

After the milk is coagulated, the formed curds are left undisturbed for 5 min making sure that the temperature does not fall below 63°C. After this, the formed curd was separated from the whey using a hoop and muslin cloth (Bhattacharya et al., 1971). Proper maintenance of these conditions is important to obtain acceptable textural and organoleptic properties in paneer.

2.1.4.7 Hooping and pressing

The separated curds are moved onto a hoop, with muslin cloth and are pressed to give the paneer a solid shape and remove the excess moisture. According to Khan and Pal (2011), various researchers have suggested different pressures for different durations, to obtain paneer of suitable shape and moisture content. Bhattacharya et al. (1971) and Sachdeva et al. (1991) used a pressure of 40-45 kg for a period of 10-15 min on a hoop of 35×28×10 cm for manufacturing paneer from buffalo milk having a moisture content of 56 %. Kumari and Singh (1992) used 0.08 kg/cm² pressure for the preparation of cow and buffalo milk paneer having a moisture content of 47.9 % and 42.7 % respectively. While researchers like Aneja et al. (2002) suggested the use of 70–100 kg weight on hoops for a period of 10-15 min for the manufacturing of paneer.

2.1.5 Different additives used in the manufacturing of paneer

Various additives have been used for the manufacturing of paneer, in order to improve several quality characteristics of paneer and to minimise production cost, increase yield and shelf life.

2.1.5.1 Calcium compounds

The addition of calcium chloride (CaCl₂) is considered beneficial for the manufacturing of paneer as it helps the formation of protein cross-linkages, thereby increasing the milk solid content and giving a better body and texture (Khan & Pal, 2011). CaCl₂ at the rate of 0.08-1.5 % has been found to produce cow milk paneer of better quality (Sachdeva et al., 1991). Singh and Kanawjia (1988) reported that the use of 0.1 % calcium chloride improved the yield, sensory properties, and total solids recovery of paneer. Calcium helps to neutralise the protein charges in milk and thus helps the precipitation of casein (Guo et al., 2003). The use of calcium chloride with high temperature also helps in the co-precipitation of whey and casein protein which in turn helps to increase the yield of paneer (Hill et al., 1982).

2.1.5.2 Vegetable oil

Oils like hydrogenated vegetable oil and ground nut oil were used for the manufacturing of paneer due to low cost, low calorie, and health benefits (Khan & Pal, 2011). Paneer made by the addition of skim milk and Vanaspati (hydrogenated vegetable oil) also resulted in a product of satisfactory quality (Kanawjia & Singh, 2000).

2.1.5.3 Sodium compounds

According to Chawla et al. (1987) addition of 0.1 % sodium citrate or 0.5 % sodium chloride improved the moisture content and hence the yield of low-fat paneer. The addition of 0.5 % sodium chloride in milk improved the body and texture of low-fat buffalo milk paneer and increased the storage life (Yadav et al., 1994). It was also observed that immersing the paneer in 1-5 % salt solution reduced the moisture content and water activity while increasing the overall flavour of the paneer (Kaur et al., 2003).

2.1.5.4 Hydrocolloids

Sachdeva and Singh (1988a) found that the addition of 0.10, 0.15 % hydrocolloids like pre-gelatinised starch, carrageenan, and sodium alginate, resulted in paneer with increased moisture content, hence higher yield. Another study by Roy and Singh (1994) observed that the incorporation of 0.1 % pre-gelatinised starch with high coagulation temperature enhanced the body and texture of filled paneer. Carboxymethyl cellulose was used as an additive by Sharma et al. (1999) in deep-fried paneer and observed a considerable reduction in oil absorption by the paneer.

2.1.6 Different variations of paneer

2.1.6.1 Protein-enriched paneer

Various studies have been done to improve the nutritional quality of paneer using non-conventional sources of protein, thus making it ideal for people suffering from protein deficiency and heart-related issues (Khan & Pal, 2011). Singh et al. (1991) reported that the addition of whey solids increased the yield by 20.9 % while decreasing the recovery of milk solids. Salve (2007) suggested the use of 2.0 % whey protein concentrate to enhance the quality of low-fat paneer made with buffalo milk having 4 % fat.

2.1.6.2 Low fat paneer

Due to the high-fat percentage of regular paneer, paneer containing lower levels of fat had been manufactured for the much health-conscious people (Kumar et al., 2014). Good quality paneer was developed by National Dairy Research Institute, Karnal (India) using milk with 3.0 % fat, (Kanawjia & Khurana, 2006). It was observed by Kanawjia and Singh (2000) that the incorporation of soy solids to skim milk enhanced the rheological and sensory properties of paneer, along with reducing the cost of production.

2.1.6.3 Soy milk incorporated paneer

Due to the high cost associated with milk and milk products, soy protein has been used to produce products of low cost and high nutritional value. Babje et al. (1992) studied the changes in the characteristics of buffalo milk paneer with the addition of soy milk. The results of their study showed that up to 20 % soy milk could be added to buffalo milk without any negative effect. The resultant paneer was similar to milk paneer in terms of springiness, color, and taste. Further, the quality of soy paneer could be improved by the incorporation of sodium caseinate. Other varieties of paneer like herb-incorporated and fiber-enriched paneer were manufactured by Kaur et al. (2003), Bajwa et al. (2005), and Kanawjia and Khurana (2006).

2.1.7 Texture of paneer

Texture of food is an important characteristic contributing to the overall sensory quality of food. It is the structural arrangement of particles within a food substance. Compared to other quality parameters, texture is highly sensitive and unstable to techniques like freezing, cold storage, thawing, cooking, and other processes. It influences the customer's food habits and shelf life of the food (Jain & Mhatre, 2009). Various terms like chewy, soft, hard, weak, fragile, coarse, pasty, mealy, and rubbery have been used to explain the texture of paneer (Arora & Mital, 1991; Patil & Gupta, 1986; Sachdeva & Singh, 1988b). Texture of paneer is very important during cooking as it should be firm enough for cutting and slicing, and soft enough for crushing during mastication (Jain & Mhatre, 2009).

Desai et al. (1991) investigated the textural properties of paneer obtained from six different sources and observed a significant difference in hardness, gumminess, and chewiness, and only a negligible difference in cohesiveness and springiness. They found that hardness was inversely proportional to moisture content while, calcium content being directly proportional in paneer. It was also reported by Kumari and Singh (1992) that the paneer made using buffalo milk had higher hardness and springiness values in contrast to the paneer made using cow milk, which had higher cohesiveness, chewiness, and gumminess properties.

Karadbhajne and Bhoyarkar (2010) studied the changes in the texture of buffalo milk paneer with the addition of various coagulants over a period of 8 days stored at 4-6°C. The results showed that 2 % and 4 % ascorbic acid had better yield, colour, flavour, and taste while 2 % ascorbic acid showed good firmness and springiness properties. Sour fruit juices were used as coagulants by Ahmed and Bajwa (2019) and the results showed that even though the paneer obtained exhibits good functional properties, it lacked various textural properties. Soy protein

isolate (SPI), in the concentration 0, 0.1, 0.2, and 0.3 % was used as fat replacers in 4 types of paneer made from low-fat milk (3 % milk fat (MF) and 10 % solid-not fat (SNF) by Kumar et al. (2011) and was compared to the one with high fat paneer made with high-fat milk (6 % MF and 9 % SNF), the results showed that increasing concentration of SPI increased the physico-chemical properties like protein, moisture, ash and also the yield while decreasing the fat, moisture protein ratio and the calorie content. The low-fat paneer samples had higher gumminess, firmness, and chewiness values than the control high-fat paneer.

Jain and Mhatre (2009) studied the textural properties of soy paneer by blending different proportions of soy milk and dairy milk at different coagulating temperatures. The results obtained showed an initial decrease in hardness, cohesiveness, and chewiness with the increase in soy milk from 0 % to 50 %, and a slight increase in these properties with the increase in soy milk from 50 % to 100 %. Several researchers like Babje et al. (1992) and Shere et al. (1991) reported that the addition of soy milk to buffalo milk up to 20 % during the manufacturing of paneer had no significant negative effect on the quality and was similar in colour, taste and springiness to milk paneer. Addition of soy milk or groundnut milk to cow milk has resulted in paneer with a weak body, high moisture content, and low textural properties when compared to paneer made from cow milk alone (Uprit & Mishra, 2004). Texture profile analysis of paneer reported by several researchers is given in Table 3.

Table 3: Texture profile analysis data of various paneer reported in previous studies

Product		Hardness (N)	Cohesiveness	Chewiness	Springiness	Reference
Milk paneer	Citric acid	12.650	0.497	0.493	0.77	(Ahmed & Bajwa, 2019)
	Lemon juice	23.1	0.467	0.839	0.77	
	Amla juice	18.8	0.413	0.680	0.86	
Buffalo milk 3 % fat		12.272	0.678	5.525	0.66	(Uprit & Mishra, 2004)
Cow milk 4 % fat		10.11	1.53	94.69	–	(Kapoor et al., 2021)
Cow milk 3 % fat		16.18	1.067	1.349	0.99	(Jain & Mhatre, 2009)

2.2 Milk

2.2.1 Introduction

Milk is a complex biological fluid produced by female mammals with varying concentrations of components like proteins, lipids, vitamins, and minerals which are essential for the growth and development of their young offspring (Bhat et al., 2016). Milk, especially bovine milk has been consumed by human civilisations for ages and has played a major role in their nutrition (Evershed et al., 2008; Wijesinha-Bettoni & Burlingame, 2013). The nutritional benefits of dairy and dairy-related products have been well researched and established by the Food and Agriculture Organisation (FAO) and other government agencies worldwide (Wijesinha-Bettoni & Burlingame, 2013). Proteins are necessary for the growth and development of the human body as it provides the essential amino acids necessary for the production of various hormones, enzymes, transport carriers, etc (IOM, 2005). About 30 % of the total dietary protein is delivered by milk and milk products in countries like the US, Canada, Australia, New Zealand and other European countries (McSweeney & Fox, 2013). Milk by itself is consumed as a beverage around the world and also used as an additive in hot beverages like tea and coffee. It is used for the manufacturing of various processed foods like confectionery and bakery. Milk is also the major ingredient for the production of dairy-based products like cheese, butter, yoghurt, ice-creams, etc (Chandan, 2008).

2.2.2 Composition of milk

The major constituents of milk are water, proteins, fat, and lactose, it also contains trace levels of other components like hormones, vitamins, minerals, enzymes, and various other compounds (Boland & Singh, 2019).

Water is the major component of milk, and it acts as a medium where other components are present in suspended or dissolved form. During the process of cheese making, most of the water from milk is lost in the form of whey. The remaining moisture is necessary for the growth of various microorganisms, which will play a crucial role in the development of texture, flavour, and colour of the final product (Chandan, 2008). Lactose is the major carbohydrate present in milk and is made up of glucose and galactose linked by a β 1-4 glycosidic bond. The major function of lactose is to provide energy to the young offspring (Boland & Singh, 2019).

Milk fat is a mixture of triglycerides, and the remaining 1-2 % of milk fat is phospholipids, carotenoids, steroids, and fat-soluble vitamins like A, D, E, K. In the milk, fat exists in the form

of small globules which are about 1-20 μm in size. Fat globules are generally surrounded by proteins that help to stabilise the emulsion and prevent the fat from sticking to each other and hence separating due to its low density (Chandan, 2008).

2.2.3 Milk proteins

Proteins in milk are of two types, whey proteins, and caseins, they are present either in their dissolved state or as colloidal suspensions. Of the total proteins present in milk, caseins make up to 80 %, and the remaining 20 % is composed of whey proteins (Chandan, 2008). Figure 2 represents the composition of milk proteins.

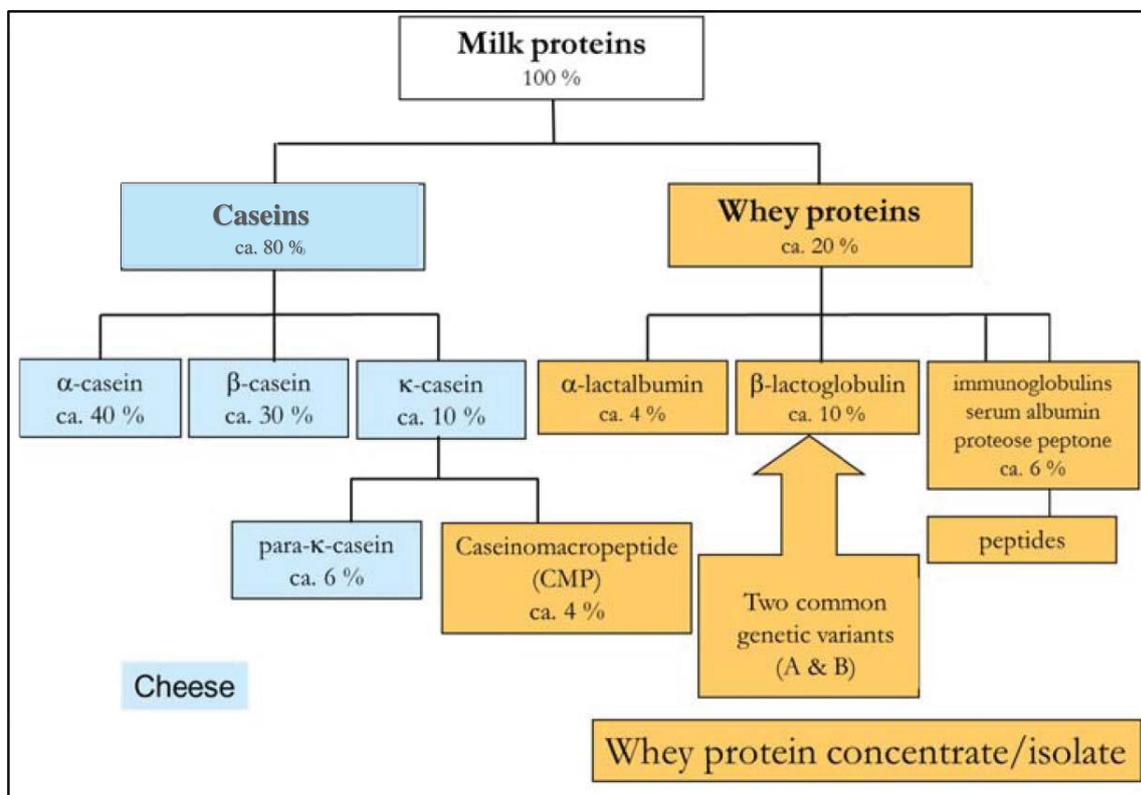


Figure 2: Composition of milk proteins (Cheison & Kulozik, 2017)

2.2.3.1 Caseins

Caseins can be defined as the protein, that is precipitated from milk at a pH of 4.6. They are unique to milk and have various specific properties. Caseins are major sources of calcium, phosphate, and other amino acids necessary for the growth of young mammalian offspring and also performs the biological function of transporting calcium phosphate through the mammary milk system without calcification (De Kruif & Holt, 2003).

Caseins can be further divided into 4 different types: α -s1, α -s2, β - caseins and κ -caseins (Walstra et al., 2006), and it accounts for 12-15, 3-4, 9-11 and 2-4 g/L of the total caseins present in milk, respectively (McSweeney & Fox, 2013). Various genetic variations have also been found for all caseins. All caseins are phosphorylated while κ -caseins are mostly glycosylated. Among the various caseins α -s1, α -s2 and β - caseins are calcium sensitive while κ -caseins are insensitive to calcium. Furthermore, 2 other types of caseins, γ , and λ caseins have also been identified, which are the results of hydrolysis of β -caseins and α -s1 caseins respectively, by proteinases and plasmin's present in milk. These proteins lack a proper structure, resulting in their stability. They are hydrophobic in nature and have a high charge due to the presence of proline and few cysteine residues (McSweeney & Fox, 2013). The different kinds of proteins are discussed below.

2.2.3.1.1 α -s1-casein

It represents about 40 % of the total casein protein present in bovine milk. It has a molecular mass of 23.0 kDa before phosphorylation and about 23.6 kDa after phosphorylation. It has a pI value ranging from 4.4 to 4.8 (Huppertz, 2013). It is made up of 199 amino acid residues with 16 Serine amino acid residues, of which 8 are phosphorylated (Mercier et al., 1971). Various studies have been done to understand the secondary structure of α s1-casein. A study done by Byler and Susi (1986) had not found any secondary structure in α s1-casein while various other studies have found different degrees of secondary structures in α s1-casein (Huppertz, 2013).

2.2.3.1.2 α -s2-casein

It constitutes about 10 % of the total casein present in bovine milk (Huppertz, 2013). It exhibits different levels of intermolecular disulphide linkages and phosphorylation (Rasmussen et al., 1994; Swaisgood, 1992). It has 207 amino acid residues with a molecular mass of 24.3 kDa which increases up to 25.2 kDa during the phosphorylation of 11 Serine residues (Huppertz, 2013).

2.2.3.1.3 β -casein

It constitutes about 35 % of the total casein in bovine milk and contains about 209 amino acid residues having a molecular mass of about 23.6 kDa which increases to 24.0 kDa during phosphorylation of 5 Serine residues. It has a pI value of 5.1 which reduces to 4.7 during phosphorylation. It is amphipathic in nature resulting in various properties (Huppertz, 2013). They are hydrophobic and have a high concentration of proline residues (Walstra et al., 2006).

2.2.3.1.4 κ -casein

It is quite different from other types of casein proteins and shows unique properties. It is the smallest of all caseins and is insensitive to calcium. κ -Casein has low levels of phosphorylation and is the only type of casein that occurs in glycosylated form. It has 169 amino acid residues and has various levels of phosphorylation (Huppertz, 2013). This protein has 2 cysteine residues that form intramolecular disulphide linkages, and exists in milk as oligomers consisting of 5-10 monomers, and has a molecular weight of about 120 kDa (Walstra et al., 2006).

2.2.3.2 Casein micelles

Casein in milk exists in the form of special structures known as casein micelles. They are spherical and are colloidal in nature. All the different types of casein (α -s1, α -s2, β , κ) are held together by colloidal calcium phosphate (CCP). They are stabilised by various non-covalent bonds into large globular aggregates in the ratio of approximately 4:1:3.5:1.5 (α -s1: α -s2: β : κ) (Dalglish & Corredig, 2012; Huppertz et al., 2018). The size of casein micelles in bovine milk ranges from 50-500 nm with an average of 120 nm. Bovine milk contains 10^{14} - 10^{16} micelles per 1 mL of milk and is quite tightly packed within a distance of 2 micelles diameter. Properties of casein micelles are given in Table 4. Casein micelles are 94 % protein on a dry matter basis and the remaining 6 % constitute of CCP, which includes calcium and phosphate along with small amounts of magnesium, citrate, and other trace elements. The white colour of milk is due to the scattering of light by casein micelles. Milk loses its white colour when the CCP, which holds the casein micelles together is dissolved by citrates, EDTA, oxalate or by increasing the pH or by the addition of urea (>5M) or ethanol (35 % at 70°C) (O'Mahony & Fox, 2013).

Table 4: Average properties of casein micelle (O'Mahony & Fox, 2013)

Characteristic	Value
Diameter	130-160 nm
Surface	8×10^{10} cm ²
Volume	2.1×10^{-15} cm ³
Density (hydrated)	1.0632 g/cm ³
Mass	2.2×10^{-15} g
Water content	63 %
Hydration	3.7 g H ₂ O/g protein
Voluminosity	4.4 cm ³ /g
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^4 Da
Number of peptide chains (MW: 30,000 Da)	10^4
Number of particles per mL milk	10^{14} - 10^{16}
Whole surface of particle	5×10^4 cm ² /mL milk
Mean free distance	240 nm

Understanding the casein micelle structure is necessary for enhancing its processing stability and monitoring its functional properties (Li & Zhao, 2019). Studying the structure of casein micelle has been the focus of many researchers for ages (O'Mahony & Fox, 2013). The earliest attempt to understand the structure of casein micelle was done by Waugh (1958). Various models have been proposed to understand the internal and external structure of casein micelles, which includes the sub-micelles model (Walstra, 1990), nanocluster model (Horne, 2006), and the dual binding model (Dalglish, 2011). In all these models proposed, it is common to find κ -caseins arranged towards the surface of casein micelles. It is responsible for the stability of casein through steric and electrostatic stabilisation (De Kruif & Zhulina, 1996).

The structure of casein micelles is highly sensitive to variations under different environmental conditions like pH, ionic strength, and temperature, and these changes can modify their various functional properties like gelling, emulsification, and foaming (Broyard & Gaucheron, 2015).

2.2.3.3 Whey proteins

Whey proteins constitute the remaining 20 % of milk proteins (O'Mahony & Fox, 2013), and are the by-product of cheese-making (Cheison & Kulozik, 2017). It is soluble at pH 4.6 and is also known as serum protein. Some of the phosphopeptides in whey proteins are derived from casein (O'Mahony & Fox, 2013). Whey proteins comprise of β -lactoglobulin, α -lactalbumins, proteose-peptones, small amounts of protein derived from blood, immunoglobulins, and serum albumin. Whey proteins are majorly globular proteins with uniform distribution of polar, non-polar, and charged residues. Intramolecular folding takes place in these proteins due to the formation of di-sulphide bonds among the cysteinyl residues which results in the positioning of hydrophobic residues towards the inner side of the molecule, thus, these proteins do not aggregate heavily nor react with other proteins in their natural state (Chandan, 2008).

Whey proteins consist of two major types of proteins: lactalbumin and lactoglobulin. Lactalbumins are soluble in 50 % saturated $(\text{NH}_4)_2\text{SO}_4$ or saturated MgSO_4 , whereas lactoglobulins precipitate under this medium (Boland & Singh, 2019). Lactoglobulins consist majorly of immunoglobulins while the lactalbumins consist of two important proteins; β -lactoglobulin and α -lactalbumin, along with other minor proteins like blood serum albumin and lactoferrins (O'Mahony & Fox, 2013). Whey proteins are less sensitive to calcium and more sensitive to heat compared to casein (Kinsella & Morr, 1984).

2.2.3.3.1 β -Lactoglobulin

β -Lactoglobulin represents about half of the proteins present in whey and about 12 % of total proteins in bovine milk (O'Mahony & Fox, 2013). They are the principal proteins present in whey and are globular in nature. β -lactoglobulin in bovine milk consists of 162 amino acid residues for every monomer, with a molecular weight of 18 kDa, it has an isoelectric pH of 5.2 (O'Mahony & Fox, 2014). At room temperature β -lactoglobulin exists as a dimer of two non-covalently linked monomeric molecules (Cheison & Kulozik, 2017).

2.2.3.3.2 α -Lactalbumin

It constitutes about 20 % of the total whey proteins in bovine milk. It is small, about molecular weight of 14 kDa, well defined, and has a globular structure formed by 123 amino acid residues which are stabilised by four disulphide bonds (Cys6-Cys120, Cys61-Cys77, Cys73-Cys91, and Cys28-Cys111). It has a sulphur content of 1.9 %. α -Lactalbumin has a high concentration of tryptophan (4 residues per mole), resulting in its specific absorbance at 280 nm. It has no phosphate or cysteine (sulphydryl group) and has an isoionic point of pH 4.8 and least solubility at a pH of 4.8 in 0.5M NaCl (Cheison & Kulozik, 2017; O'Mahony & Fox, 2013).

The primary structure of α -lactalbumin is homologous with lysozyme. 54 out of the 123 amino acid residues present are identical to the amino acid residues in egg white lysozyme and 23 others are structurally similar. α -Lactalbumin is believed to be evolved from lysozyme through gene duplication. α -Lactalbumin is a part of lactose synthetase. It is the enzyme that catalyses the terminal step in the biosynthesis of lactose. α -Lactalbumin is a metalloprotein consists of two Ca^{2+} for every molecule in a pocket containing 4 Aspartic acid (Asp) residues. Calcium-containing bovine α -lactalbumin is the most stable to heat of the major whey proteins, as they renature themselves after denaturation by heat. When the pH is dropped to less than 5.0, the Asp residue loses its ability to bind Ca^{2+} as it gets protonated (O'Mahony & Fox, 2014).

2.2.3.3.3 Blood serum albumin

Bovine milk has low levels of Blood serum albumin (BSA) ($0.1\text{-}0.4\text{ gL}^{-1}$; 0.3-1.0 % of total nitrogen), mostly due to leakage from blood. It has no known biological function in milk. BSA forms heat-induced intramolecular disulphide linkages with α -lactalbumins and β -lactoglobulin thus, influencing denaturation, gelation, aggregation properties of β -lactoglobulin. It has no or little effect on the properties of milk ingredients like whey protein concentrates and isolates due to its low concentration (O'Mahony & Fox, 2013).

2.2.3.3.4 Immunoglobulins

Milk of mature bovine consists of 0.6 - 1 gL⁻¹ Ig (~3 % of total nitrogen). Immunoglobulins are a very complex set of proteins. They are divided into 5 different types: IgA, IgG (IgG1 and IgG2), IgD, IgE, IgM. Of these various types, IgG, IgA, and IgM exist in milk. IgG consists of two large and two small polypeptide chains bonded by disulfides.

Immunoglobulins consist of 2 units bonded by a junction component and a secretory component whereas IgM contains 5 bonded four chain units. The major function of Ig is to provide immunity to the newborn offspring (O'Mahony & Fox, 2013).

2.3 Gelation of milk proteins

Milk protein gelation is the main phenomenon behind the development of cheese and other fermented milk products (Lucey, 2020), which is one of the most popular and oldest kinds of foods and occupies an important position in the food products market. The gel structure in these products is a result of coagulation of casein micelles which makes up 80 % of the total proteins in milk (Li & Zhao, 2019). Milk protein gels are usually irreversible in nature compared to many polysaccharide gels which are thermoreversible in nature. Casein micelles are the major proteins responsible for the development of protein networks. They are held together by hydrophobic interactions and calcium phosphate nanoclusters around the phosphoserine clusters (Lucey & Horne, 2018). Gelation of milk proteins can be induced in a number of ways like heat treatment, enzyme addition (rennet), and acidification (acids). Various dairy products are also produced by a combination of one or two of these methods, for example, quark cheese which is normally a cultured product made by adding small quantities of rennet (Lucey, 2020). The method of gelation and the processing technique used, like heat treatment, affects the digestion and the release of amino acids in the milk gels (Barbé et al., 2013). Paneer is an example of a combination of heat and acid coagulation. Since paneer is not rennet-induced gel, effects of rennet on milk proteins will not be discussed here and more focus will be given to acid and heat-induced gelation of milk proteins.

2.3.1 Heat-induced coagulation of milk proteins

Heat treatment is the most standard process applied for the processing and manufacturing of milk and other dairy-related products. Casein micelles are usually stable to high temperatures due to the absence of a proper tertiary structure, while whey proteins are quite sensitive to thermal processing. Whey proteins above 70°C expose their reactive thiol group which then

interacts with other whey proteins or caseins through thiol-disulphide linkages to form a combination of casein micelles coated with whey proteins and aggregates of whey proteins (Donato & Guyomarc'h, 2009). Whey proteins coated to the surface of casein micelles are highly pH dependent. About 30 % of the denatured whey proteins attach to the surface of casein micelles at pH 6.7 and it increases to about 75 % when it is heated at pH 6.3 (Anema & Li, 2003). According to Raikos (2010), all these changes are irreversible in nature. During heating, the interior structure of casein may get altered while the basic structure of casein, in general, remains stable. At a pH greater than 6.5, heating of milk can cause wide separation of κ -, α - and β -caseins (Anema & Klostermeyer, 1997; Donato & Dalgleish, 2006).

In addition to changes in protein, heating also causes major losses in soluble calcium and phosphate which are most likely converted to their colloidal phase (Anema et al., 2011; Blecker et al., 2012).

2.3.2 Acid-induced coagulation of milk proteins

Native casein micelles, at the natural milk pH, are stabilised by their net negative charge and steric repulsion (Lucey & Singh, 2003). Glucono Delta Lactone (GDL) or starter cultures are commonly used for the acidification of milk (Li & Zhao, 2019). As the pH reduces, the net negative charge on the casein micelles decreases, causing the κ -casein (hair-like projection) on the surface of casein micelles to collapse (De Kruif, 1997), thereby reducing the electrostatic repulsion and steric stabilisation. Further decrease in pH causes colloidal calcium phosphate to solubilise (Heertje et al., 1985) causing the casein particles to aggregate. Lucey (2017) described three stages of milk acidification based on pH ranging from 6.7 to 4.6.

1) pH 6.7 to 6.0 - As the pH decreases from 6.7 to 6.0 there is a decrease in the net negative charge of casein micelles thus lowering the electrostatic repulsion. The structural changes of casein micelles remain unchanged as only a small quantity of CCP is dissolved above pH 6.0.

2) pH 6.0 to 5.0 - The lowering in pH further reduces the net negative charge of casein micelles along with electrostatic repulsion. The hairs present on the surface of κ -casein are charged and as the pH decreases, the hairs may reduce or disintegrate resulting in the lowering of net charge due to a decrease in electrostatic and steric stabilisation. Almost all the CCP present inside the casein micelles are dissolved around pH 5.0.

3) pH <5.0 - At pH below 5, it reaches its isoelectric point where net negative charge decreases and there is an increase in Van der Waals forces (+/- interaction of charges). There

is an increase in hydrophobic interaction due to a decrease in electrostatic repulsion (Horne, 1998, 2001).

In the case of milk that is not heat-treated, the gelation pH is about 4.9, while in the case of milk that is heat-treated, the gelation pH is much higher. Casein proteins come together and aggregate due to charge neutralisation during acidification. As a result of this, there is a change in the charge density of the system and the electrostatic repulsion / Van der Waals attraction balance also gets modified (Mezzenga & Fischer, 2013). Acidification results in the development of chains and clusters which come together to form a 3-D network (Kalab et al., 1983; Lucey, 2017).

Acidification of milk can be done in two ways; fermentation by the addition of microbes which is usually slow, or chemical acidification by the addition of acids like citric acid, GDL, etc. Chemical acidification is generally done for the manufacturing of various cheeses like cottage cheese as it is faster. Milk gels formed by acid-induced coagulation differ from milk gels produced by bacterial cultures in their rheological and structural properties (Lucey et al., 1998; Renan et al., 2008). An increase in storage modulus has been observed during the cooling of gels likely due to swelling of casein by the poor hydrophobic interactions and due to increased surface area within the particles (Lucey, 2017). The charged groups on the surface of caseins are removed by the high ionic environment and hence causing poor interaction between casein particles (Lucey, 2020).

2.4 Global protein challenge

2.4.1 Introduction

Food provides the major components essential for the survival of human beings and has played a pivotal role in the existence of mankind. Of the major components present in food, proteins play a significant role in the growth and development of our body.

A total of 20 amino acids can be found in the nature of which 11 are known as non-essential amino acids and the rest 9 are called essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) (Nadathur et al., 2017). Figure 3 gives a representation of amino acids and the flow of dietary proteins in the body. Other than its nutritional importance, proteins are also used greatly by food industries as an ingredient during food formulations due to their numerous functional properties (Toldrá et al., 2018).

Due to its amphiphilic nature and structural flexibility, food proteins can bind with other components of food like carbohydrates, other proteins, vitamins, and minerals through various interactions like electrostatic interactions, hydrogen bonds, and/or hydrophobic bonds (Quintero et al., 2017).

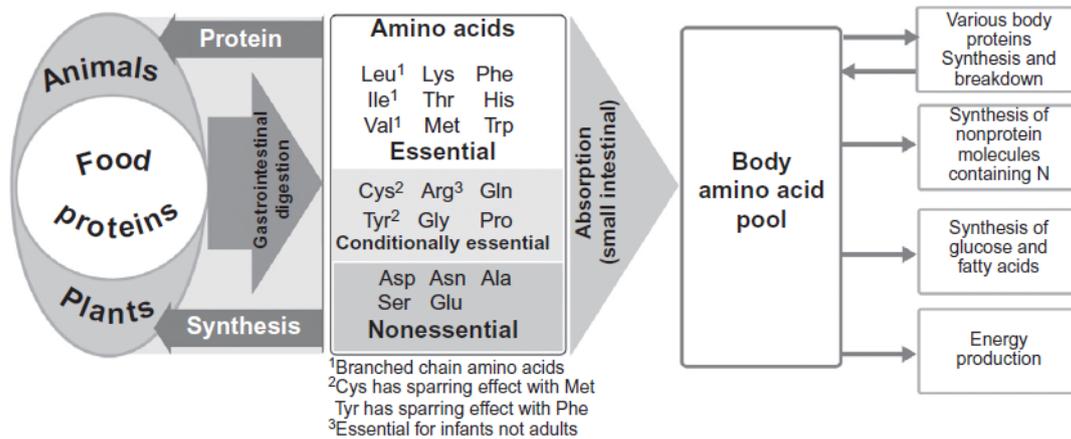


Figure 3: Representation of amino acids and the flow of dietary proteins (Nadathur et al., 2017)

The global population is estimated to grow to about 9.6 billion by 2050 thus raising the demand for animal-based protein (UN, 2015). Due to the increase in novel market income, there is a huge demand for proteins, i.e. an estimated increase in demand of 58 % for dairy, 74 % for meat, and about 500 % for eggs (Euromonitor, 2017).

The production of animal proteins puts a lot of pressure on the environment and requires larger areas and other resources when compared to plant-based proteins. Furthermore, it also causes the emission of greenhouse gases. The increasing concern over the issues of sustainability and food security have significantly affected the food choices and lifestyle of customers and have led to the creation of various groups like flexitarians, who focuses on reducing the consumption of animal protein without completely removing it from the diet (Dagevos & Voordouw, 2013; UN, 2015).

Due to the huge rise in population, there is a rising concern among the industries as well as government agencies regarding feeding the growing population. Globally meat production is a major source of protein and a lot of resources are utilised for its production. There is a need for the use of plant-based proteins as an alternative to animal proteins (Nadathur et al., 2017). The production of animal-based proteins puts a huge pressure on the environment as it requires

about 100 times the water required for the production of a similar quantity of plant-based proteins (Sapkota et al., 2007).

There has been a gradual decline in the use of dairy-based products as it is known to cause various allergic reactions in certain individuals and also due to various health concerns like antibiotic residues in milk and cholesterol. The decline is also due to consumer awareness regarding the negative impacts of the dairy industry on the environment (Jeske et al., 2018).

Food production causes an emission of a significant amount of greenhouse gases (GHG) and food industries are responsible for about 29 % of global warming in the European countries (Huppes et al., 2006). According to FAO, 18 % of the world's GHG emission occurs due to livestock, of which 4 % comes from dairy manufacturing industries (Steinfeld et al., 2006). Meat and dairy production generates high GHG and requires more land area on a per kg basis when compared to plant-based foods (Nijdam et al., 2012; Sonesson et al., 2010). Figure 4 gives a depiction of various consequences on the environment at various stages of food production.

Another major sustainability issue regarding the use of animal protein is the vast area of land used permanently for the pasture of animals. This land usage is almost 20 times greater than cropland and up to 3 times bigger than arable land. According to FAO reports, of the total cropland, 33 % is used for the production of livestock feed (Euromonitor, 2017).

Every year, 13 billion hectares of forest land are converted into agricultural land for various purposes like crop production and production of feeds for livestock. Thus, putting extra pressure on the soil fertility, availability of water, and biodiversity, which further results in the severe effect of climate change. The development and utilisation of new sustainable sources of protein for both humans and animal fodder are very important and can help to take the pressure away from the land and other resources (Euromonitor, 2017).

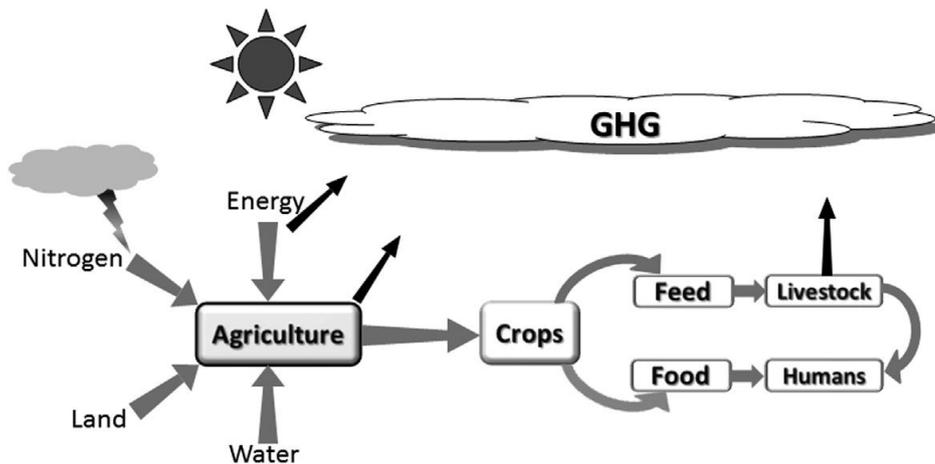


Figure 4: Depiction of various consequences on the environment at various stages of food production (Nadathur et al., 2017)

Thus, the use of plant-based proteins to replace animal proteins partially or completely can have a positive impact to mitigate the negative impacts of the loss of biodiversity and climate change (Fasolin et al., 2019)

2.4.2 Plant-based sources of protein

Plant-based sources of proteins include various legumes, nuts, pulses, seeds, and vegetables. The production of plant proteins is more efficient and eco-friendlier, as it requires minimal usage of various resources like water, land, and fossil fuels compared to the production of animal-based proteins (Nadathur et al., 2017). It also has a lesser impact on the environment in terms of land use and the emission of GHG. Another important advantage of plant-based proteins is the low cost and wide availability compared to animal proteins. All these factors have influenced the utilisation of plant proteins as a feasible protein source (Henchion et al., 2017). However, animal proteins are superior to plant-based proteins in terms of protein content as plant proteins lack certain essential amino acids (Lonnie et al., 2018). The nutritional quality of a protein cannot be determined by its essential amino acid content alone, other factors like digestibility and bioavailability, which affect its utilisation in the body also need to be considered (Lynch et al., 2018). A combination of plant proteins in the right proportion can help to create products with well-balanced amino acid profiles and such products are encouraged by the Academy of Nutrition and Dietetics (Melina et al., 2016). It is well understood that the consumption of plant-based proteins has a positive effect on the gut microbiota, reduced risk of cardiovascular diseases and type 2 diabetes (Busnelli et al., 2018; Malik et al., 2016; Song et al., 2016; Tharrey et al., 2018). Due to these various benefits, there

has been a rapid increase in works exploring the nutritional and functional properties of plant-based proteins (Fasolin et al., 2019).

A change in dietary preferences to plant protein-based foods can feed up to 10-20 times more humans compared to animal proteins. About 20 times more soy protein can be produced from the same land in contrast to the quantity of beef produced. Similarly, 13 times the quantity of rice and 10 times the amount of legumes can be produced from the same land (Nadathur et al., 2017). Therefore, there is a need for better utilisation of plant-based proteins in order to minimise the negative impact of animal-related protein on the environment.

2.4.3 Pulses as a sustainable source of protein

Pulses (peas, chickpea, lentil, bean) are a suitable alternative to animal-based proteins especially due to their high protein content (18-32 %), health benefits, and affordability (Boye et al., 2010). Pulses are ideal for enhancing the diet of people especially in the poorer regions of the world. They are 5 times cheaper compared to milk. Some varieties of pulses like soybeans, lupini beans, lentils, and fava beans have a very high content of proteins. They are high in nutrients and are suitable for people with health concerns like gluten and lactose intolerance (Euromonitor, 2017). Numerous health benefits such as lower risk of cardiovascular diseases, cancer, hypertension, diabetes, osteoporosis, adrenal disease, gastrointestinal disorders, and reduction of low-density lipoprotein (LDL) cholesterol are associated with the consumption of pulses (Hu, 2003; Jacobs & Gallaher, 2004; Tharanathan & Mahadevamma, 2003). Pulse proteins usually consist of globulins and albumins. Globulins are salt soluble and are the major type of proteins present in pulses. It consists of 11S- legumin and 7S vicilin. 11S legumin consists of a hexameric quaternary structure with acid and basic subunits. On the other hand, 7S vicilin has a trimeric structure with a molecular mass of 175000 – 180000. Albumins are present in lower concentrations and are water-soluble. It consists of enzymatic proteins, protease inhibitors, amylase inhibitors, and lectins and has a molecular mass of 5,000 - 80,000 Da. Covicilin, a third type of storage protein has also been purified from pea seeds. Pulses also contain minor amounts of proteins like prolamins and glutelins (Boye et al., 2010).

Other than their nutritional and health benefits, proteins in pulses are also known for their functional properties (Table 5) like solubility, water binding capacity, foaming, etc, that are useful in food formulations and processing (Boye et al., 2010). There is a rapid increase in the

use of legume protein isolates in the food industry for the manufacturing of meat analogues, milk substitutes, and gluten-free dairy products (Santos-Hernández et al., 2020).

Table 5: Various functional properties of proteins (Nadathur et al., 2017)

Functional Property	Mechanism and Physicochemical Property of Protein	Example Food System	Example Protein	Sensory Property
Solubility	Hydrophilicity, charge and ionization of surface residues, H-bonding	Milk, protein-rich beverages, nondairy milks	Dairy, soy, almond, rice, proteins	Flavor, taste, mouthfeel, turbidity
Viscosity	Hydrodynamic size and shape, H-bonding	Soups, gravies, salad dressings, desserts	Gelatin, soy, egg	Taste, consistency, mouthfeel
Water binding	H-bonding, ionic hydration	Comminuted meats, low-fat meat products, bakery products	Muscle, egg, cereal, soy proteins	Texture, consistency
Gelation (heat-induced)	Water entrapment and immobilization, network formation, thermal aggregation	Emulsified meat products, bakery products, puddings	Muscle, egg, dairy, and many seed proteins	Mouthfeel, texture, grittiness, smoothness
Cohesion and adhesion	Hydrophobic-, ionic- and H-bonding	Emulsified meats, pasta and noodles, bakery products, extruded snacks	Muscle, egg, dairy, and several seed proteins	Stickiness, chewiness, particulate
Elasticity	Hydrophobic bonding, disulfide cross-linking	Meat products, leavened bakery products, extruded products	Muscle proteins, gluten protein, casein	Texture, crispiness, chewiness
Emulsification	Adsorption and film formation in oil–water interface, hydrophobicity and hydrophilicity	Comminuted meats, cakes, soups, salad dressings, nondairy milks, desserts	Muscle, egg, dairy, and several seed proteins	Mouthfeel, flavor, smoothness
Foaming	Adsorption and film formation in air–water interface, hydrophilicity and hydrophobicity	Ice cream, cakes, whipped toppings, mousses, desserts	Dairy, egg, and certain seed proteins	Mouthfeel, smoothness, frizziness
Fat and flavor binding	Hydrophobic bonding entrapment	Flavored milks, protein-rich beverages, emulsified meats, bakery products, sauces and gravies	Dairy, egg, muscle, and many seed proteins	Flavor, odor, smoothness

Soybean is one of the most commonly used plant proteins and is used as a common ingredient in a variety of food products like milk alternatives, ice-creams, yoghurts, creams as well as meat substitutes. Due to its high allergenicity and prevailing sceptical nature, there is an increasing concern among the health-conscious generation for the use of soy-based products, thus, there is a need for better and a more sustainable source of plant proteins (Euromonitor, 2017).

Currently, plant-based products in the market mainly focus on soy and almond-based products which also contribute to certain environmental problems. Due to the huge demand for soy, there is a rapid expansion of soybean plantations to various sensitive ecosystems like rainforests and savannahs which lead to the extinction of various plants and animals thus, playing a crucial role in climate change. Similarly, the production of almonds requires a large

amount of water. Therefore, there is a need for the use of more sustainable plant-based sources of proteins like peas, peanuts, quinoa, etc (Fearnside, 2001; Jeske et al., 2018).

Food allergy is also a major concern among people around the world. The major 8 allergens, which require labelling in countries like the USA, Canada and the European Union include soybean, peanut, gluten-containing products, tree nut, milk, eggs, fish and shellfish. Although pulses like beans, pea, chickpea and lentils are found to be allergenic in nature, they are not classified as major allergens (Boye et al., 2010).

2.4.4 Mixing plant and animal protein to improve the techno-functionality

The huge demand for animal protein and the resulting environmental damage can be minimised by partially substituting animal proteins with plant proteins in various food formulations (Henchion et al., 2017; Messin et al., 2017). An increasing number of research groups have shown interest in the development of products or ingredients based on animal proteins by partially replacing them with plant proteins (Chihi et al., 2016; Messin et al., 2017), although creating such a product without changing its physicochemical and sensory properties still remains a huge challenge (Amagliani & Schmitt, 2017; Chihi et al., 2016; Lin et al., 2017; Messin et al., 2017; Silva et al., 2019).

Plant proteins alone as an ingredient has a very distinct taste and poor water solubility, but are associated with low cost of production, low allergenicity (except soy and gluten), and unique functional properties (Chihi et al., 2016; Day, 2013; Karaca et al., 2011; Lin et al., 2017; Silva et al., 2019). On the contrary, dairy proteins are superior to plant-based proteins, both in the case of nutrition and digestibility. Thus, the use of plant protein alone as dairy product alternatives is not practical and viable. Therefore, partially replacing milk proteins with plant proteins improves the scope for developing food products with higher nutritional value, desired properties, and low cost, in a sustainable manner (Silva et al., 2019).

Studying the mechanism behind plant and animal protein interaction can open the doors to develop innovative applications and new markets (Alves & Tavares, 2019). Recently, there has been a lot of research regarding the development of food products or ingredients with the partial substitution of animal proteins with plant proteins (Chihi et al., 2016; Messin et al., 2017; Silva et al., 2019). Several studies have suggested that mixed protein systems are very efficient in adjusting protein gel textures and to form stable emulsions and foams (Alves & Tavares, 2019).

The article by Alves and Tavares (2019) suggests several possibilities of plant and animal protein mixtures that can be used for the development of innovative products (Figure 5). These include the potential to; 1) control the size of thermal-induced protein aggregates, 2) improve the overall protein content of the gels without significant rheological alterations, and 3) reduce the cost of production. Their review also mentions the lack of studies on plant-based proteins other than pea and soy, and on animal proteins other than milk proteins.

The production of plant-based dairy substitutes can create new markets and act as a substitute for dairy products (Jeske et al., 2018). Ouyang et al. (2021) investigated the potential of proteins to be used as an ingredient to develop cheese with characteristics sensory properties tailored towards southeast and east Asian customers. Cheese with a soft texture and mild-milky flavour is generally preferred by Asian buyers. They also suggested the use of proteins common to the Southeast and East Asian population as it may be easier for them to accept familiar flavours. Due to its several health benefits mung bean is used as a functional food in China and several other Asian countries. Therefore, the developed cow milk-mung bean protein paneer can be a potential product for the East and Southeast Asian markets.

One of the major unanswered questions is the digestibility of plant and animal protein mixture. Knowing the difference between the kinetics of protein digestion in a single system and a mixed system is essential for the creation of products with high nutritional and biological value (Alves & Tavares, 2019).

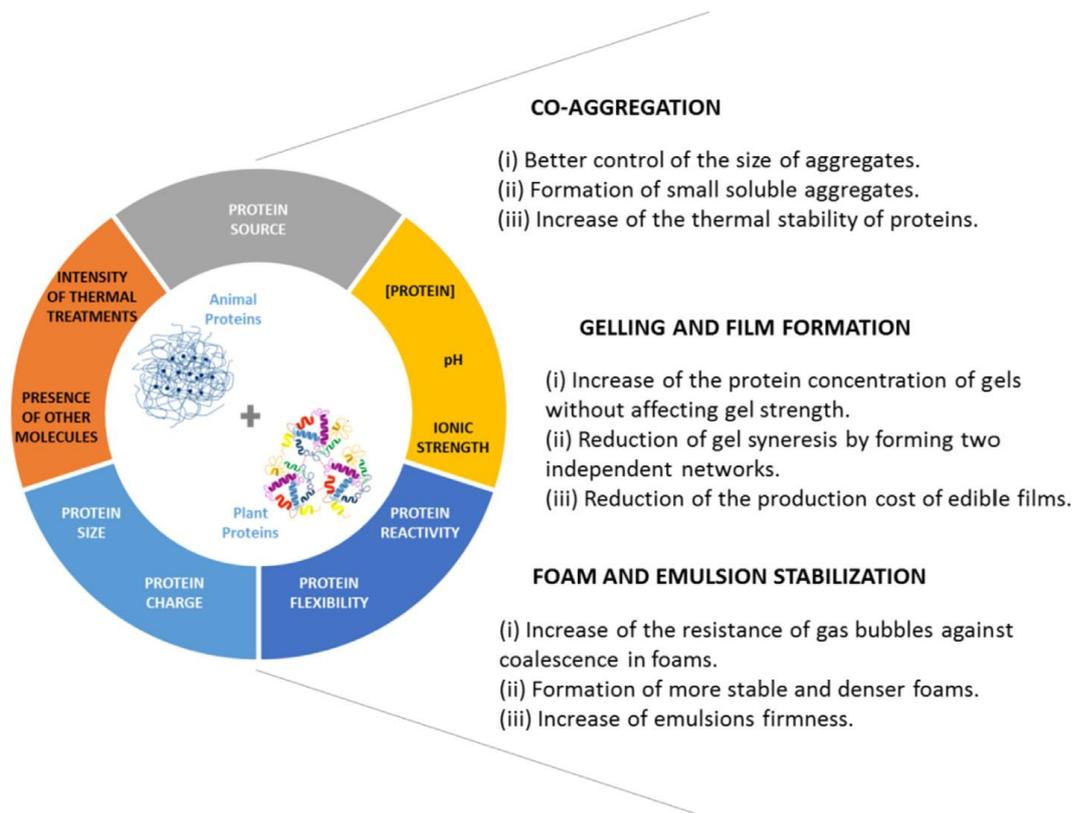


Figure 5: Representation of the major factors affecting plant-animal proteins in a mixed system and their functionalities (Alves & Tavares, 2019)

2.5 Mung bean

2.5.1 Introduction

Mung bean (*Vigna radiata*) belongs to the legume family and has been cultivated widely around southeast Asia, Australia, South America, and Africa. It has been cultivated in China for more than 2000 years and has been consumed in Asia as a snack, bean sprouts, or bean noodles (Brishti et al., 2017; Du et al., 2018). It is a summer crop and has a short growth period of (70 - 90 days). It is also grown in the warm regions of Canada, United States, and Southern Europe (Yi-Shen et al., 2018). Mung bean is grown in more than 6 million hectares of land worldwide and is cultivated widely as it requires low input and is moderately drought-tolerant (Hou et al., 2019).

Mung bean is rich in nutrients and has a high concentration of iron, manganese, calcium as well as vitamin B and C. It has a protein content of about 20 – 27 %, a low-fat content, and has good digestibility compared to proteins of other legumes (Brishti et al., 2017; Fan & Sosulski, 1974; Yi-Shen et al., 2018). Proteins from mung seeds are considered to be biologically active

(Henry & Kettlewell, 2012). Mung bean is also referred to as "green pearl" due to its high protein content (Brishti et al., 2017).

There is a huge rise in the demand for plant proteins especially due to the increasing cost of animal-derived proteins and concerns regarding health and food security (Du et al., 2018). Since mung bean protein is a good and cheap source of protein, it can be a good option for the health-conscious population (Brishti et al., 2017). It has a good amino acid composition and matches well with the composition of soybean protein and FAO/WHO reference protein (Fan & Sosulski, 1974; Thompson, 1977). Due to its low glycaemic index and ability to stop LDL oxidation, it is a good option for people suffering from diabetes. The protein hydrolysates and bioactive peptides present in mung bean protein prevent the angiotensin converting enzymes and can control blood and heart diseases (Aluko, 2008).

In addition, mung bean has also shown to have antioxidant, antifungal, and anti-tumour effects (Wang et al., 2004). The extraction of mung bean is mainly done for its starch and usually, the protein left is discarded as the by-product, which can be used as a potential source of protein (Du et al., 2018; Wang et al., 2016). It has an adequate amount of essential amino acids except methionine, cysteine, and tryptophan. The lysine concentration in mung bean is similar to that of eggs (Coffmann & Garciaj, 1977; Siemensma et al., 1993).

Mung bean protein has a lower concentration of sulphur-containing amino acids, therefore it is advised to consume mung bean with cereals which are a high source of sulphur-containing amino acids except for lysine (Yi-Shen et al., 2018). As a result of its high protein composition and hypoallergic nature, it is also used as an ingredient for the manufacturing of infant foods (Bazaz et al., 2016; Dahiya et al., 2014).

The presence of antinutritional factors in mung bean can limit the absorption of essential nutrients, for instance, phytic acid can bind with various divalent cations like iron, zinc, calcium, and magnesium forming insoluble complexes which are hard to digest thus limiting its absorption and utilisation. Other antinutritional factors present in mung bean are tannins, trypsin inhibitors, proteinase inhibitor, polyphenols and hemagglutinins (Dahiya et al., 2015; Sandberg, 2002). The antinutritional factors can be further reduced or removed by various techniques like cooking, dehulling, germination, and fermentation (Barakoti & Bains, 2007; Hou et al., 2019).

2.5.2 Proximate composition

Proximate composition is essential for determining the overall quality and to study the potential to be used as a food product or ingredient (Brishti et al., 2017).

Dahiya et al. (2015) studied the nutritional and technological properties of mung bean and gave the maximum and minimum values reported for each chemical constituent (Table 6).

The major chemical components present in mung bean are protein, fat, carbohydrate, fibre, ash, fatty acids, and amino acids while minerals and vitamins are present in minor quantities.

Table 6: Proximate composition of mung bean reported in different studies (Dahiya et al., 2015)

Macronutrient	Average*	Minimum	Maximum	References
Moisture (g/100 g)	9.80	4.10	15.20	(Kadwe et al., 1974; Watson, 1977; Tsou and Hsu, 1978; Khatoon and Prakash, 2004; Mubarak, 2005; Khattak et al., 2007a; Sampath et al., 2008)
Crude protein (g/100 g) dm	23.8	14.6	32.6	(Kadwe et al., 1974; Watson, 1977; Tsou and Hsu, 1978; Rao and Belavady, 1979; Shehata and Thannoun, 1980; Rao and Deosthale, 1983; Khader and Rao, 1986; Ignacimuthu and Babu, 1987; Prabhavat, 1990; Poehlman, 1991; Singh and Singh, 1992; Sathe, 1996; Jood et al., 1998; Bravo et al., 1999; El-Adawy et al., 2003; Khatoon and Prakash, 2004; Mubarak, 2005; Fatima and Kapoor, 2006; Barakoti and Bains, 2007; Khattak et al., 2007a, 2007b; Mallillin et al., 2008)
Crude lipid (g/100 g) dm	1.22	0.71	1.85	(El-Adawy et al., 2003; Khatoon and Prakash, 2004; Mubarak, 2005; Fatima and Kapoor, 2006; Barakoti and Bains, 2007; Watson, 1977; Tsou and Hsu, 1978; Shehata and Thannoun, 1980; Prabhavat, 1990; Poehlman, 1991; Singh and Singh, 1992; Sathe, 1996; Jood et al., 1998; Bravo et al., 1999)
Crude fiber (g/100 g) dm	4.57	3.8	6.15	(Watson, 1977; Tsou and Hsu, 1978; Shehata and Thannoun, 1980; Prabhavat, 1990; Poehlman, 1991; Singh and Singh, 1992; Sathe, 1996; El-Adawy et al., 2003; Mubarak, 2005; Barakoti and Bains, 2007)
Ash (g/100 g) dm	3.51	0.17	5.87	(Tsou and Hsu, 1978; Shehata and Thannoun, 1980; Rao and Deosthale, 1981; Rao and Deosthale, 1983; Prabhavat, 1990; Poehlman, 1991; Sathe, 1996; Watson, 1977; Bravo et al., 1999; El-Adawy et al., 2003; Khatoon and Prakash, 2004; Mubarak, 2005; Fatima and Kapoor, 2006; Barakoti and Bains, 2007; Khattak et al., 2007a)
Carbohydrate (g/100 g) dm	61.0	53.3	67.1	(Watson, 1977; Shehata and Thannoun, 1980; Prabhavat, 1990; Poehlman, 1991; Singh and Singh, 1992; Sathe, 1996; El-Adawy et al., 2003; Mubarak, 2005)
Energy (kcal/100 g) dm	344	338	347	(Poehlman, 1991)

*Mean value of all collected data.

From Table 6, a huge variation in the data can be observed which may be due to the different varieties of mung bean, different growth conditions, and different methods used for the analysis. Majority of the researchers have used the micro-kjeldahl method with a conversion factor of 6.25 for protein estimation while Cai et al. (2002) have used combustion to convert nitrogen in their samples to nitrogen oxide, reducing to nitrogen and detecting it using a thermo conductivity detector.

Table 7: A comparison of the proximate composition of mung bean flour, mung bean protein isolate and soy protein isolate (Brishti et al., 2017)

	Mung bean flour	Mung bean protein isolate	Soy bean protein isolate
Protein (%)	23.84 ± 0.04 ^a	81.53± 0.02 ^b	86±0.86 ^c
Fat (%)	1.53 ± 0.04 ^a	0.14±0.01 ^b	0.11±0.01 ^b
Fiber (%)	4.95 ± 0.08 ^a	0.73±0.02 ^b	0.10±0.00 ^c
Ash (%)	3.02 ± 0.03 ^a	4.38±0.28 ^b	3.47±0.05 ^c
Moisture (%)	10.21 ± 0.06 ^a	4.56±0.11 ^b	4.56±0.05 ^b
Carbohydrate (%)	56.43 ± 0.16 ^a	8.66±0.26 ^b	5.76±0.88 ^c

#Each value in the table represents the means; ±SD of triplicate determinations ^{a-c} Means with the different superscript letters in the same row indicated significant difference (p < 0.05).

Moisture, protein, and fat content in mung bean flour reported by Brishti et al. (2017) (Table 7) are similar to the results obtained by Blessing and Gregory (2010). Higher protein contents of 25 % and 27.5 % were reported by Butt and Rizwana (2010) and Mubarak (2005) which may be due to the differences in the production environment, agricultural practices, and different cultivators (Abbas & Shah, 2007).

In the case of protein isolates, even though there is a significant difference in the protein content of mung bean protein isolate (81.53 %) compared to soy protein isolate (86 %), it is still higher than several other legumes. No major difference can be observed in the fat and moisture content of these two protein sources, while, fibre, ash, and carbohydrate contents have been observed to differ (Brishti et al., 2017).

2.5.3 Mung bean protein

Mung bean contains 24-28 % proteins (Li et al., 2010; Liu et al., 2015) and of the total proteins present, 85 % are storage proteins which include albumins, globulins and prolamins (Chen et al., 2014; Shewry et al., 1995). Globulins and albumins make up about 60 and 25 % of the total mung bean proteins. The remaining 15 % of the mung bean proteins have not yet been studied greatly except for thiamine-binding proteins (Gunarti et al., 2013), trypsin inhibitor (Klomklao et al., 2011), and non-specific lipid transfer proteins (nsLTP) (Wang et al., 2004). Table 8 shows the different types of mung bean protein along with their molecular weight.

The three types of globulins present in mung bean proteins are identified as basic type vicilin (7S), vicilin type (8S), and legumin type (11S) globulins. It comprises about 3.4 %, 89.0 %, and 7.6 %, respectively.

and 7.6 % of the total mung bean globulin composition (Mendoza et al., 2001; Yi-Shen et al., 2018).

8S vicilin or globulin which comprises 89 % of the total globulins is the major storage protein present in mung bean. It is further composed of four subunits having molecular weights of 60, 48, 32, and 26 kDa. Mendoza et al. (2001) found the N-terminal amino acid sequence of the four subunits, which are EDKEEQ (60 kDa), IDAAEVSVSRGKNNPFYFNN (48 kDa), SKTLSSQNEPFLRLN (32 kDa), and IDGAEVSVSRGKNNP (26 kDa) (Yi-Shen et al., 2018).

Liu et al. (2015) performed SDS-PAGE of the globulin fraction of mung bean protein under reducing and non-reducing conditions and observed that under reducing conditions the globulin protein showed four bands with molecular weights 61000, 50000, 32000, and 26000, while under non-reducing conditions, there was no change in the four bands which indicates the absence of disulphide linkages in 8S globulin due to lack of cysteine amino acid. Similar results were found by Mendoza et al. (2001) and Tang and Sun (2010). Di-sulphide linkages are important factors affecting the functional properties of mung bean (Liu et al., 2015).

Further, it has been understood that the 8S globulin in mung bean has 3 isomeric forms 8S α , 8S α' , and 8S β , which shows about 90 % sequence identities. The 8S globulin present in mung bean has a high level of sequence and structural similarity with β -conglycinin (7S globulin) present in soybean. Therefore, 8S globulin in mung bean may have similar properties to 7S globulin in soybean (Yi-Shen et al., 2018). It was also observed that the 11S globulin present in mung bean under SDS-PAGE shows 2 bands having a molecular weight of 41000 and 20000, but under the non-reducing conditions, these bands disappeared indicating the presence of disulphide linkages (Liu et al., 2015).

Only very few studies have reported the characteristics of 7S and 11S globulin present in mung bean protein. Mendoza et al. (2001) reported using SDS-PAGE that, the two bands resulting from 11S and 7S globulins are 40 kDa, 24 kDa and 28 kDa, 16 kDa in size.

Table 8: Protein fraction of mung bean along with their molecular weight (Yi-Shen et al., 2018)

Types	Proteins	Subunits	Molecular weight
Storage proteins (85% in overall proteins)	Globulins (60% in overall proteins)	Globulin 7S	28kD
			16kD
			8S _α 5.2kD
		Globulin 8S	8S _α 5.2kD
			8S _β 5.2kD
		Globulin 11S	40kD
		24kD	
	Albumin (25% in overall protein)		
Other proteins (15% in overall proteins)	Trypsin inhibitor		14kD
	Non-specific lipid transfer peptide (nsLTP)		9.03kD
	Thiamine-binding proteins (TBP)		72.6kD
	Others		

2.5.4 Nutritional properties and amino acid profile

Mung bean has a total protein content of 24-28 % (Li et al., 2010) and has a chemical score of 76 %, calculated based on the WHO and FAO guidelines by Mubarak (2005). MBPI has a total protein content of 87.8 %, with a total amino acid content of 800.2 mg/g (Table 9). Of the total amino acids 43.5 % constitute of essential amino acids, of which approximately 1.6 % consists of sulphur-containing amino acids. Mung bean contains a sufficient amount of most essential amino acids, except methionine and cystine. MBPI are particularly rich in essential amino acids like leucine, lysine, and phenylalanine/tyrosine followed by valine, isoleucine and histidine. The aromatic amino acid composition in MBPI was 12.1 % of which phenylalanine and tyrosine constitute 11.3 %, i.e. 90.3 of 800.2 mg/g (Yi-Shen et al., 2018).

Glutamic acid and aspartic acid were reported to show the highest values in MBPI. It is also abundant in amino acids like glutamine and arginine which are believed to be essential for the development of the brain and showing neuroprotective functions in infants (Keunen et al., 2015). Dietary glutamine can enhance gastrointestinal barrier integrity by lowering consistent infections and by stimulating lymphocyte proliferation, monocyte function, and T cell 1 helper cytokine response that may enhance brain development. In addition to that, arginine has been

shown to increase nitric oxide production and cerebral blood flow to help decrease the occurrence of necrotising enterocolitis (Yi-Shen et al., 2018).

It can be clearly observed that the total essential amino acid content in MBPI surpasses the recommendations given by WHO/FDA (FAO, 1991). However, the levels of threonine, tryptophan, and total sulphur-containing amino acids (methionine and cystine) are insufficient (Yi-Shen et al., 2018).

Due to the deficiency in sulphur-containing amino acids, mung bean protein is similar to other legume proteins in terms of nutritional quality (Bernardo et al., 2004). To address this problem several researchers have used protein engineering as a solution. Torio et al. (2011) were able to successfully incorporate methionine into 8S α globulins of mung bean protein, thereby increasing the nutritional quality of modified mung bean proteins (MBP), in terms of amino acid score from 41% to 145 %. Another report by Torio et al. (2012) used protein engineering to incorporate sulfhydryl groups and di-sulphide linkages to create cystine-modified 8S α mung bean globulin.

The review by Hou et al. (2019) showed that mung bean has health benefits like hypoglycemic and hypolipidemic effects along with anti-cancer, antihypertensive, anti-melanogenesis, hepatoprotective, and immunomodulatory activities. Mung bean also contains a sufficient amount of nutrients and bioactive compounds, mainly polyphenols, polysaccharides, and polypeptides and it also offers various pharmacological benefits.

Table 9: Amino acid composition of MBP with reference to guidelines given by WHO/FAO (Yi-Shen et al., 2018)

MBPI levels	Amino acids	MBPI (mg g ⁻¹)	FAO/WHO (mg g ⁻¹)
Overview	Total amino acids	800.2	
	Total essential amino acids	348.2 (43.51%) ^a	
	Total aromatic amino acids	96.7 (12.08%) ^a	
	Total sulfur amino acids	13.0 (1.62%) ^a	
Higher levels	Phenylalanine + Tyrosine	90.3	63
	Leucine	74	66
	Lysine	62.4	58
	Valine	46.3	35
	Isoleucine	39.1	28
	Histidine	27.9	19
Lower levels	Threonine	28.4	34
	Methionine + cysteine	13	25
	Tryptophan	6.4	11
Not mentioned by the FAO/WHO	Glutamic acid/glutamine	125.4	
	Aspartic acid/asparagine	85.3	
	Arginine	64.4	
	Serine	38.5	
	Alanine	36.6	
	Glycine	32.2	
	Proline	30	

MBPI, mung bean protein isolates.

^aPercent of amino acids, relative to total amino acids in MBPI.

2.5.5 Functional properties of MBP

Proteins are abundantly used as ingredients during food processing due to their various physicochemical properties and therefore, it is necessary to understand these properties of MBP to be used as an ingredient (Tang et al., 2009). Several research reports have been published regarding the various physicochemical properties of mung bean, such as emulsion stability, emulsifying activity, foaming capacity, foam stability, protein solubility, oil binding capacity, water binding capacity, and thermal properties (Yi-Shen et al., 2018). Thus, the use of such proteins or peptides can improve the quality of food, for instance, proteins with good emulsifying properties help to stabilise emulsions, foams, and beverages better thus, improving its shelf life (Liu et al., 2015). Table 10 includes the different functional properties of MBPI.

Studies done by Du et al. (2018) concluded that MBP can be utilised as a good source of high-quality protein with the potential to be used as a nutraceutical and a food ingredient. Brishti et al. (2017) compared the functional properties of mung bean protein along with soy protein and observed that both isolates had good protein solubility in both acidic and alkaline pH (pH 2 and pH 8-2) compared to pH 4. Other functional properties like gelation, foaming, water holding capacity, and oil holding capacity were found to be higher for mung bean protein

isolates than soybean protein isolates. Liu et al. (2015) studied the functional properties of 8S globulin fraction of the mung bean protein from 15 different mung bean cultivators and found that they varied considerably depending on the variety and also showed improved results compared to 7S protein in mung bean.

Table 10: Functional properties of MBPI (Yi-Shen et al., 2018)

	Protein solubility (%)	Water absorption capacity	Oil absorption capacity	Emulsion activity (%)	Emulsion stability (%)	Foam capacity (v/v, %)	Foam stability (v/v, %)	thermal properties
MBPI	–	3.33 ± 0.57 g g ⁻¹	3.00 ± 0.00 g g ⁻¹	63.18 ± 0.38 ^a 72.03 ± 0.53 ^b	62.75 ± 0.43 ^a 66.50 ± 1.37 ^b	89.66 ± 0.57	80.83 ± 1.04 ^c	157.90 ± 0.17°C ^d
MBPI	–	2.62 ml g ⁻¹	10.5	–	–	26	76.9	–
MBPI	61.6 ± 1.5/65.6 ± 2.1 ^a	–	–	–	–	–	–	100.8 ± 1.1°C ^d
MBPI	70.6	–	–	–	–	–	–	–
MBPI	72 ± 4.44	163 ± 10.05%	113 ± 6.84%	41.10 ± 1.87	21.00 ± 1.29	110 ± 6.78	58 ± 3.58	–
Globulins	92–99	–	–	90–120	–	–	–	80.8–83.0°C ^d
8S globulin	51–75	1.37–2.14 g g ⁻¹	1.90–3.84 g g ⁻¹	1.54–5.55	45–78	47–106	39–97	–

MBPI, mung bean protein isolates.

^a In distilled water; ^bin 3% NaCl; ^cafter 15 min; ^ddenaturation peak temperature; ^e untreated/heated.

2.6 Gelation of plant proteins

Protein gelation is the crosslinking of polypeptide chains to form a 3-dimensional network through various molecular linkages like hydrogen, ionic, disulphide, and hydrophobic bonds. The type of forces involved in the gel formation depends upon the type of protein, its structure, and the methods used for isolation. Many of the storage proteins can take part in a number of chemical reactions and can form various interactions like intermolecular di-sulphide linkages due to the presence of certain amino acids like cysteine and glutamine. Thus, it can form gels of various structural composition necessary for the formulation of different food products. Protein gelation is the process of conversion of proteins from their solution state to gel state by various physical and chemical techniques. Gel formation is very important in the food manufacturing industry as it imparts many new characteristics to the food matrix, like water holding capacity, flavour carriers, etc (Bröckel et al., 2013).

Gelation in proteins can be of two types: 1) gels due to random aggregation and 2) gels due to aggregation of molecules into strands in a more organised manner. Protein gelation occurs due to the unfolding and aggregation of proteins. Heating causes the exposure of the reactive groups due to denaturation, which forms intermolecular bonds with other protein molecules. A 3-D network is developed with adequate intermolecular linkages. Various factors affecting protein gelation are pH, ionic concentration, rate of cooling and heating and the molecular force (Bröckel et al., 2013).

2.6.1 Gelation of legume proteins

The effective utilisation of plant products to a greater extent depends upon the understanding of the scientific knowledge of processing and functional properties of the plant constituents. Products like tofu in particular have been developed to a greater extent. On the other hand, legumes other than soy have only shown limited applications and are usually consumed as whole seeds. One of the important ways to improve the value of legumes is through fractionating the seed to proteins and starches. However, due to lack of techniques or methodologies, these fractions have not been able to convert into final food products for human consumption (Cai & Baik, 2001).

Heating of the legume proteins above its minimal denaturation temperature causes a modification by changing its structure to form an interconnected globular structure. The required gel structure is developed by hydrophobic and hydrogen bonds, together with disulphide linkages (Brishti et al., 2017). Compared to soy protein, MBP has the ability to form a gel and retain water in a three-dimensional structure at a low concentration. Brishti et al. (2017) found that the Least Gelation Concentration (LGC) of mung bean protein was 12 % and that of soy protein was 14 %.

Several researchers have attempted the preparation of legume bean curds from field pea and winged bean. Their preparations typically involved the denaturation of protein prior to coagulation using coagulants to form the curds. The final results obtained were however unacceptable for human consumption due to undesirable colour and textural properties (Cai & Baik, 2001).

Various studies relating to plant protein-casein mixed systems are limited to soy protein isolates, peanut proteins and other plant protein isolates at a much lower level. Several studies have been done to understand the effects of soy protein on various types of cheese like mozzarella, domiati, cheddar, processed cheese as well as various fresh and soft cheeses. The cheese manufactured by substituting milk with soy protein had different properties to the ones produced from soy milk or pure milk. In general, cheese containing soy protein isolates were characterised by their fragile, crumbly, and brittle nature during biting (Lu et al., 2010).

In recent years, one of the major focuses of food science is to use innovative methods to produce novel food products with health benefits. Mung bean protein due to its higher gelling ability when compared to soybean protein can be utilised for the production of innovative food products.

2.7 Digestion of Food

2.7.1 Introduction

Digestion of food is an intricate process that is crucial for maintaining the health and welfare of human beings by extracting nutrients within the food matrix through various mechanical, chemical, and enzymatic conditions (Somaratne et al., 2020).

Digestion of food begins when the food enters the human body. It undergoes various physical and chemical processes to breakdown the food and to release the nutrients within the food matrix, which are then absorbed into the body. The availability of nutrients for intestinal absorption after food disintegration is called bioavailability. Various factors like mechanical properties and internal composition of the food matrices influence digestion by affecting the rate of matrix breakdown and biochemical behaviour during gastrointestinal conditions (Ayala-Bribiesca et al., 2016; Verhoeckx et al., 2015).

The human digestive system or the gastrointestinal tract (GIT) consists of the oral cavity, the oesophagus, the stomach, the small intestine including the duodenum, jejunum, and ileum, large intestine, ascending transverse, descending colon, rectum, and anus. The two major functions of the GIT are the mixing and peristaltic movements. It allows the food to move forward into the next stage of digestion. During this process, the food is mixed with various digestive enzymes and acids which breakdowns it into smaller particles. The released nutrients and compounds are then absorbed by the walls of the small intestine, from where it is transferred into the bloodstream and finally reaches different parts of the body (Singh et al., 2015).

2.7.2 Three stages of food digestion

2.7.2.1 Oral phase

This is the initial stage of digestion which happens in the mouth, and during this phase, the food is exposed to physical (mechanical force and temperature) and biochemical (pH, enzymes, mucilin, dilution, salts) processes (Foegeding et al., 2011; Sarkar et al., 2009). The food is broken down by mastication and mixed with saliva, forming into a lubricated mass known as bolus (Singh et al., 2015). Mastication helps to reduce the size of the food particles to about 0.82 - 3.04 μm depending on the nature of the food (Foegeding et al., 2011; Jalabert-Malbos et al., 2007), thus allowing it to be swallowed easily. The degree of food breakdown in the oral phase depends on the structural and mechanical properties of the food. α -Amylase is an

important enzyme present in human saliva which helps in the breakdown of starch present in the food (Singh et al., 2015; Singh et al., 2009).

2.7.2.2 Gastric phase

The bolus is then transferred from the oral cavity into the stomach where it is mixed with many gastric juices and enzymes, which causes the swelling of the food materials. Penetration of the gastric juices into the food components causes acid and enzymatic hydrolysis. These effects along with continuous mechanical shearing result in the breakdown of the food matrix, thus increasing the surface area. The lower pH of the stomach does not favour the hydrolysis of protein, but it helps in the hydrolysis of pectin which is the major component of the plant cell wall thus, softening it in the process. Pepsin is an important enzyme present in gastric juice which has a broad spectrum of activity and a preference for hydrophobic residues (Luo et al., 2015; Singh et al., 2015).

2.7.2.3 Intestinal phase

From the stomach, the bolus is further transferred to the small intestine, where it is further disintegrated (Bornhorst & Singh, 2012). The small intestine can be divided into 3 compartments: the duodenum which receives the digestive fluids from the liver and pancreas, and two other compartments jejunum and ileum. In the duodenum, the bolus is neutralised with sodium bicarbonate (NaHCO_3) to a neutral pH to improve the activity of enzymes. Pancreatic enzymes and other digestive enzymes work together for the disintegration of food components. The released nutrients are then absorbed through the inner side of the small intestine by the mechanism of simple diffusion, facilitated diffusion, or active transport (Guerra et al., 2012). Figure 6 shows a graphical representation of *in vitro* digestion of paneer.

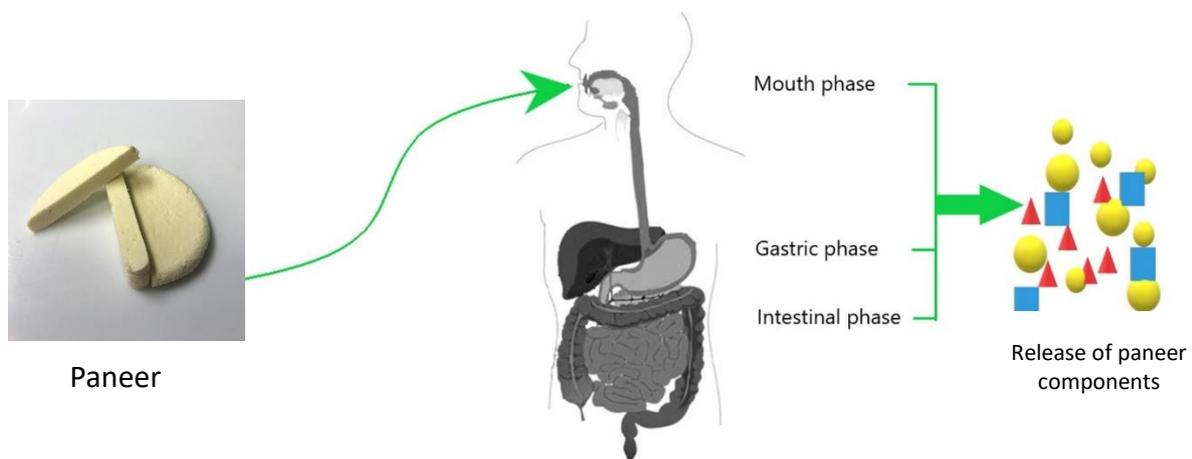


Figure 6: Graphical representation of *in vitro* digestion of paneer (modified from Žolnere et al. (2019))

2.7.3 *In vitro* digestion experiments

In vivo digestion experiments are considered to be the golden standard when studying the nutritional quality of food proteins. But, these studies come with various disadvantages such as high cost, high time consumption, and various ethical concerns (Minekus et al., 2014). In order to address this issue, *in vitro* digestion models closely resembling the physiological activities during human digestion were developed. The COST INFOGEST protocol was developed by more than 200 scientists from 32 countries having expertise in the field of digestion and was first published in 2014 (Minekus et al., 2014) and was further updated in 2019 (Brodkorb et al., 2019).

In vitro digestion is a relatively fast and cheaper way of studying the digestion of food and is a powerful tool used by researchers to investigate the effect of its various physicochemical properties on digestion (Bohn et al., 2018).

The two types of *in vitro* simulation models are the static and the dynamic models. In the static simulation model, the pH, volume, and hydrodynamic mixing remain constant within the simulation. On the contrary, the dynamic gastric simulation model involves the continuous addition of gastric juices, cycling gastric forces, and gastric emptying, thus creating a more realistic environment similar to the human digestive tract (Kong & Singh, 2010).

2.7.4 Role of food matrix during digestion

The role of food matrix on the digestibility of food is often not considered while estimating its nutritional value. Food matrix can have a great influence on the rate and food disintegration and absorption during digestion (Turgeon & Rioux, 2011).

The structure of food plays a major role in the digestion and transport of nutrients within the human body. The understanding of the degradation of food in the digestive system is important for the designing of new food products with improved functionalities (Singh et al., 2015).

In the gastric phase, the easy diffusion of the digestive enzymes and fluids on the food particles is highly dependent on the structure and properties of the food matrix (Thevenot et al., 2017).

The various structural changes happening within the gastrointestinal tract affect the release and absorption of nutrients (Figure 7). According to Bornhorst et al. (2015), the initial hardness of food is used for predicting the rate of food disintegration and digestion. For example, foods like carrot having a high initial hardness and a lower rate of texture softening have shown a lower rate of disintegration while, foods having lower initial hardness and a higher rate of

softening have shown greater rate of disintegration and absorption (Kong & Singh, 2009; Kozu et al., 2014).

Digestion and the rate of disintegration of the cheese greatly depend on its structure and its composition. For example, in the case of cheddar cheese with reduced fat, the cheese matrix is very compact as only a few spaces are occupied by fat globules thus increasing the hardness and lowering the cohesiveness compared to a regular cheddar cheese. Similarly, in the case of low-fat mozzarella cheese, due to high protein density and low-fat concentration, the cheese produced has higher hardness, cohesiveness, and springiness (Rudan et al., 1999). Thus, the higher density of the protein network affects the disintegration of cheese and its digestion in the GIT (Fang et al., 2016b).

There is a lack of information available on the structural changes of cheese in the human digestive system while the structural changes happening during digestion in natural foods like potato, carrot and peanut is highly available (Do & Kong, 2018).

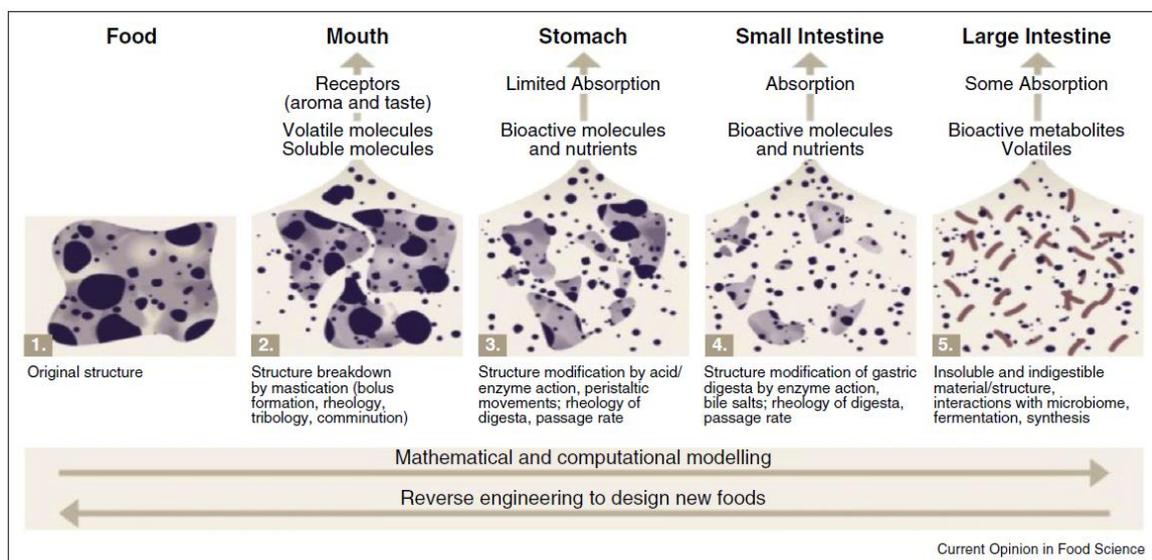


Figure 7: Graphical representation of digestion on the structural breakdown of food under different phases (Singh et al., 2015)

2.7.5 Factors affecting the structure of the food

The structure of food in the digestive system greatly depends on its composition and the various processing techniques applied (Hu et al., 2010).

Various processing methods like homogenisation, fermentation, and heat treatment are commonly used for the manufacturing of dairy products. These processes have a huge impact

on the food matrix and can affect the digestion and absorption kinetics of nutrients. The rate of breakdown of the food matrix greatly influences the release of nutrients during digestion (Lorieau et al., 2018).

Homogenisation is a common technique applied during the manufacturing of milk to reduce the size of milk fat globules and to increase its stability during storage (Lamothe et al., 2017). Various *in-vivo* (Armand et al., 1999; Borel et al., 1994) and *in vitro* studies (Garcia et al., 2014) suggested that the rate of lipid digestion has improved in emulsions with a smaller size of lipid droplets, which may be due to the increased surface area, thus allowing more lipase enzyme to act (McClements & Li, 2010).

Another important technique used for improving the shelf life of milk is pasteurisation or Ultra High temperature (UHT). Various research suggests that this process affects the release of nutrients during digestion (Gallier et al., 2013). In the case of gelled foods like cheese and yoghurt, the matrix needs to be broken down for the release of nutrients thus they show a lower and slow rate of amino acid absorption when compared to liquid or semi-liquid food. Thus, the release of nutrients during digestion highly depends upon the rate of food matrix disintegration (Barbé et al., 2013; Singh et al., 2015).

2.7.6 Digestion of cheese like products

Cheese mainly consists of a milk protein network (mostly casein) where other components like water, fat, and minerals are entrapped within the protein network (Mistry & Anderson, 1993). Proteins are an important macronutrient present in cheese and can range from 3 – 40 % depending upon the type of cheese (O'Brien & O'Connor, 2004). Its digestion is aided by acids and enzymes (pepsin) present in the stomach as well as the pancreatic and intestinal enzymes present in the small intestine (Luo et al., 2015).

During enzymatic proteolysis, the proteins are broken down into polypeptides and amino acids, of which some may be of biological importance rather than being a source for protein synthesis (Walther et al., 2008). Pepsin is an important enzyme present in gastric juice and is an aspartic protease having a wide range of activity especially for hydrophobic residues (Luo et al., 2015).

The breakdown of food in the stomach is a complex process, for instance, the proteins released within the food structures could be hydrolysed into peptides. Thus, the kinetics of nutrient release could be affected by the rate of solid food disintegration. After gastric digestion, the food is transferred to the small intestine where the proteins are hydrolysed by pancreatic

enzymes like trypsin, chymotrypsin, and exopeptidases. The digested food is dissolved in the duodenal juices and is finally absorbed by the walls of the small intestine (Fang et al., 2016a).

Different types of cheese have different cheese matrix and structures due to their varying composition and processing methods applied. For example, during the pressing of hard cheeses like cheddar cheese, the moisture content is reduced thus making the cheese structure hard and compact. In a similar way, during the manufacturing of Mozzarella cheese, the kneading and stretching process applied forms specific elastic and thread-like structures (Fang et al., 2016a; Kindstedt et al., 2004).

Few researchers have reported the effects of textural properties on the disintegration and proteins-peptide release rate of cheese during *in vitro* digestion. According to Fang et al. (2016a) and Fang et al. (2016b), the breakdown of cheese was faster when textural properties like hardness, cohesiveness, and chewiness were low. Ayala-Bribiesca et al. (2016) also showed that the rate of cheese disintegration decreases with the increase in hardness.

Protein digestibility and amino acid bioavailability of acid and rennet coagulated milk gels have been studied by Barbé et al. (2014) and concluded that the rennet induced gels show slower release of amino acids when compared to acid-induced gels.

The protein digestion rate greatly affects the release of amino acids and therefore its absorption. This could influence the postprandial utilisation of proteins like protein anabolic response and the satiating effect (Fang et al., 2016b).

2.8 Analysis techniques

2.8.1 Colour analysis

The basic parameter to assess the quality of food by a consumer is the colour of the food surface (Pathare et al., 2013). Colour determines the consumer acceptance and purchase of a product (Diezhandino et al., 2016).

Since the colour of a food can be associated with other quality parameters such as flavour like sensory parameters, nutritional parameters, and visual/ non-visual defects, it is used as an indirect measure of those quality parameters. Moreover, it is very simple and fast (Pathare et al., 2013). Colour of food can be quantified in two ways: visual or subjective colour measurements and instrumental or objective colour measurements. In the case of instrumental measurements, colour is determined using either colorimeters or spectrophotometers and colour is expressed in terms of colour coordinates. Commission Internationale de l'Eclairage

(CIE)'s $L^*a^*b^*$ which was devised in 1976 is one of the most popular instrumental colour determination system. It provides more uniform colour differences in relation to human perception of differences (Pathare et al., 2013).

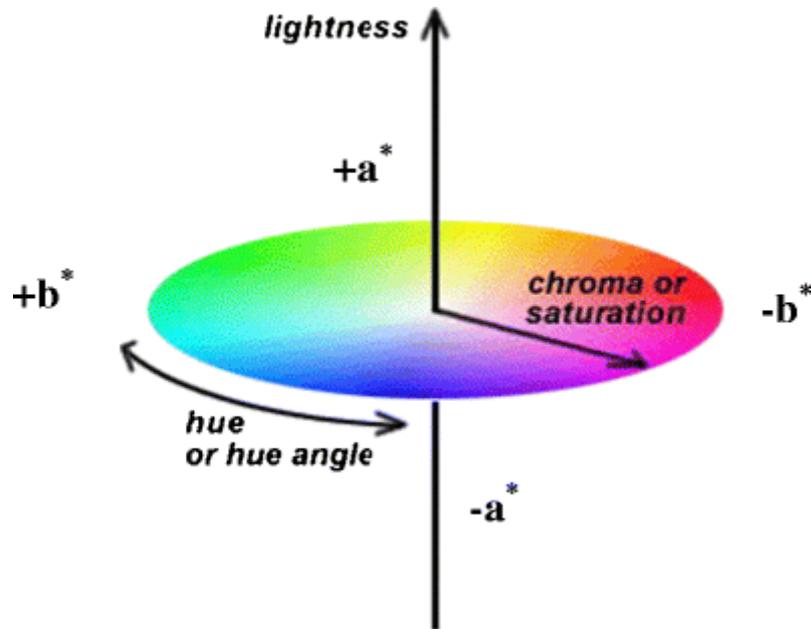


Figure 8: CIELAB colour space (Pathare et al., 2013)

The CIELAB colour space is shown in Figure 8. The three coordinates (L^* , a^* , b^*) of the CIELAB system is directly read a^* and b^* are colour coordinates and L^* is a psychometric index of lightness. The parameter a^* takes positive values for reddish colours and negative values for the greenish colours. The parameter b^* takes positive values for yellowish colours and negative values for the bluish colours. L^* is an approximate measurement of luminosity, where colour is considered as equivalent to a member of the greyscale, between black and white (Granato & Masson, 2010).

The CIELAB system has been used in several studies to determine the colour of dairy products; for example, cheese by (El-Nimr et al., 2010), (Ibáñez et al., 2016), (Okpala et al., 2010) and paneer by (Sharma et al., 2018), and (Chandravanshi et al., 2018).

2.8.2 Small amplitude rheology

Rheology can be defined as the study of the deformation and flow of matter under well-defined conditions. Rheological properties can be used to explain the behavioural characteristics and perception of various foods. A food material is composed of several components, which have a profound effect on its rheological properties. A Rheometer is generally used for determining the rheological properties of various materials like gels, suspensions, solutions, emulsions, etc. Many food materials show both viscous (liquid) and elastic (solid) properties and are therefore known as viscoelastic materials (McKenna & Lyng, 2003).

Dynamic oscillatory shear tests are carried out by treating the material to a sinusoidal deformation and calculating the outcome as a function of time. Small amplitude oscillatory shear tests are used for the analysis of food materials as it does not destroy the structure of the food material (Hyun et al., 2011). In a small amplitude oscillatory test (SAOT) the sample is placed between two parallel plates with a known gap and the upper plate is oscillated back and forth at a fixed stress or strain amplitude and frequency. The amplitude of oscillation is same as the maximum stress or strain applied and the frequency denotes the number of oscillations per min. This can be represented as a sinusoidal wave with strain or stress plotted on the y-axis and time on the x-axis (Malvern Instruments Limited, 2016) (Figure 9).

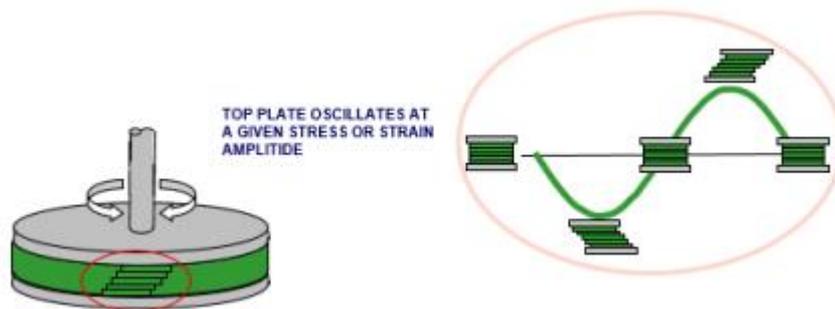


Figure 9: Diagrammatic representation showing oscillatory shear profile applied on a sample loaded between parallel geometry (Malvern Instruments Limited, 2016)

This test can be used for understanding the bonding characteristics of protein networks within the food at a molecular level when used with other analytical techniques. The relationship between complex modulus (G^*), storage modulus (G') and loss modulus (G'') is given by the equation: $|G^*|^2 = (G')^2 + (G'')^2$.

Complex modulus is the measure of sample deformation while storage modulus (G') measures the energy stored and recovered during a test cycle and loss modulus (G'') measures the energy released during a test cycle. Loss tangent ($\tan \delta$) is the ratio of storage and loss modulus (G''/G') and is an indicator of gel-solution transition point and signifies the formation or breakdown of the internal network. Commonly performed tests using small amplitude oscillatory shear analysis include strain sweeps, frequency sweeps, time sweeps, and temperature sweeps. These tests can be set up varying one or more of the experimental variables. The four major variables include stress/strain, frequency, time, and temperature (Gunasekaran & Ak, 2002; San Martín-González et al., 2007; Tunick, 2011).

Small amplitude oscillatory rheology has been used by various researchers for studying the rheological properties of cheese; low fat Iranian white cheese with different concentrations of rennet (Madadlou et al., 2005), changes in mozzarella and cheddar cheese during heating (Vogt et al., 2015), rheological properties of processed cheese (Joshi et al., 2004), development of soft cheese incorporated with soy protein isolate (Rinaldoni et al., 2014), rheological properties of whey less feta cheese incorporated with pea protein isolate (Omrani Khiabani et al., 2020).

2.8.3 Confocal microscopy

Confocal laser scanning microscopy (CLSM) is one of the most effective microscopy techniques for studying the microstructure of foods, especially dairy products. This is a powerful tool that can provide a 3-dimensional analysis of microstructure through assembling two-dimensional micrographs by computerised image analysis where the laser scanning passes through the surface of the sample to visualise thin optical sections without disturbing the internal structure (El-Bakry & Sheehan, 2014; Everett & Auty, 2008; Lopez et al., 2007). This is done by observing the specimen within a plane both transverse to and along with the optical axis (El-Bakry & Sheehan, 2014) and providing focused images up to several hundred microns in depth by an argon laser (Ong et al., 2013). This tool characterises fat globules in samples by staining protein and fat components differently. The most widely used fluorescent dyes for the characterisation of cheese microstructure are fluorescein isothiocyanate, rhodamine, and fast green for the protein phase and oil red O and Nile red for the fat phase (El-Bakry & Sheehan, 2014).

In addition to providing a 3-dimensional analysis of microstructure, CLSM possesses several advantages over conventional microscopy techniques including an improved resolution of about 0.2 μm (Everett & Auty, 2008). One major advantage is that it visualises and chemically

differentiates sample components using protein and lipid-specific stains (Ong et al., 2011). The optical sectioning capability of CLSM which avoids sample cutting artefacts at the surface is useful to examine high-fat foods, which are difficult to prepare using the conventional microscopy without the loss or migration of fat globules during sample preparation (El-Bakry & Sheehan, 2014; Ong et al., 2011). The capability of fluorescent labelling of different components within the food structure is another advantage that allows the examining of interactions and relative locations of those components within the food product, for example, bacteria in probiotic dairy products (Auty et al., 2001). However, CSLM is a relatively expensive technique due to its high cost and maintenance (El-Bakry & Sheehan, 2014).

This CLSM technique has been used in various studies to analyse the different aspects of dairy products; fat globule structure in cheese (Khanal et al., 2018), microstructure of rennet induced gel (Ong et al., 2010), milk gelation and cheese melting (Auty et al., 1999), permeability of rennet casein gels (Mellema et al., 2000), localisation of probiotic bacterial cells (Auty et al., 2001), multiple fluorescence labelling of proteins, lipids and whey in dairy products (Herbert et al., 1999), location of exopolysaccharides in cheese (Hassan et al., 2002), and correlation with sensory data of acid milk gels (Pereira et al., 2003).

2.8.4 Texture profile analysis

According to the international organisation for standardisation (ISO, 1992) texture of a food product can be defined as “all the rheological and structural (geometric and surface) attributes of the product perceptible by means of mechanical, tactile, and, where appropriate, visual and auditory receptors.” Food texture can greatly influence customer interest, buying interest, and thereby consumption (Gunasekaran & Ak, 2002). Texture of food is an important characteristic contributing to the overall sensory quality of food. It is the structural arrangement of particles within a food substance.

Texture profile analysis (TPA) is a widely used double compression test used for analysing textural properties of various food products. During this test, the food samples are compressed twice and are also referred to as the two-bite test as it mimics the biting action of the mouth (Gunasekaran & Ak, 2002). TPA has been used by various researchers for studying the textural properties of various cheese-like products, for example, shelf-life extension of paneer by Kapoor et al. (2021). Uprit and Mishra (2004) studied the textural properties of paneer fortified with soy, Ahmed and Bajwa (2019) studied the textural properties of paneer coagulated with

sour juices and Bryant et al. (1995) studied the effect of fat reduction on the textural properties of cheddar cheese.

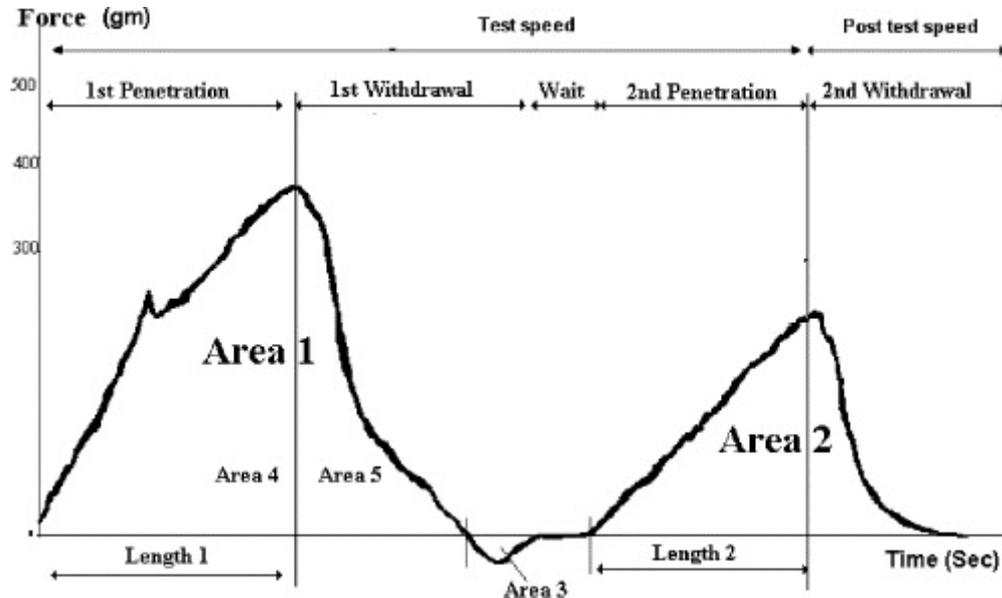


Figure 10: Texture profile analysis curve (Uprit & Mishra, 2004)

2.8.4.1 Hardness

Hardness is one of the major and commonly evaluated properties in determining the textural properties of paneer. It is explained as the force required to cause a given deformation. It is also described as the peak force of the first compression of the product during its textural analysis. The first compression indicates that during the texture analysis, the cylindrical probe is penetrated through the paneer sample causing a puncture (Jain & Mhatre, 2009).

2.8.4.2 Cohesiveness

Cohesiveness can be understood as the degree to which a material can be deformed before it gets completely broken down and it greatly depends on the internal strength of the material. It is determined as the area of the curve obtained during the second compression, divided by the area of the curve obtained during the first compression (Jain & Mhatre, 2009).

2.8.4.3 Springiness

Springiness is determined as the distance of the height of the material during the second compression divided by the original compression distance. Springiness parameter can only be

compared with products that are identical in their shape and height (Jain & Mhatre, 2009). Springiness depends upon various factors like the degree of protein unfolding, heat treatment, and protein interactions (Supekar et al., 2014).

2.8.4.4 Chewiness

Chewiness can be defined as the energy required for breaking down the food, making it easy for swallowing. It can only be done for foods that are solid in nature (Jain & Mhatre, 2009).

2.8.5 Ninhydrin reactive amino N

The ninhydrin assay is used to determine the concentration of free amino groups as a measure of protein hydrolysis. This method was used in earlier days for the identification of amino group containing compounds especially in foods, and it was introduced for the quantitative determination of amino acids in the late 1940s (Sun et al., 2006).

The principle of ninhydrin assay is as follows. Ninhydrin reacts with the α -amino group of primary amino acids and produces a dark purple compound known as Ruhemann's purple upon heating. The chromophore formed is the same for all primary amino acids and the intensity of colour depends on the number and chemical nature of the amino groups being analysed. The optimum pH for the overall reaction is pH 5.5. Absorbance measurement by UV-visible spectroscopy is made at 570 nm which is the spectral maximum of Ruhemann's purple (Field & Field, 2010; Perrett & Nayuni, 2014).

Although several instrumental methods are currently used for this purpose, ninhydrin assay still consists advantages such as simplicity, convenience, non-requirement of expensive equipment, and ability to analyse a large number of samples (Sun et al., 2006). Although the O-Phthalaldehyde (OPA) method became popular over ninhydrin assay in recent years for the determination of protein hydrolysis during digestion, it has some drawbacks compared to the ninhydrin method such as higher risk of false positives caused by ϵ -amino groups and danger associated with handling it (Reynaud et al., 2020).

In contrast, the amounts of α -amino-group N which can be identified by the ninhydrin method is lower than that of OPA or 2,4,6-trinitrobenzenesulfonic acid (TNBS) methods (Reynaud et al., 2020). Some other drawbacks include non-stoichiometric behaviour with the amount of colour formation and variability of colour with product nature (Friedman, 2004).

Ninhydrin method has been used by researchers for the estimation of amino group containing compounds in different types of samples; α -amino nitrogen in pea protein hydrolysates

(Panasiuk et al., 1998), proteolysis in ripening cheese (Pearce et al., 1988), melatonin in pharmaceutical formulations (Amin et al., 2000), collagen-like polymer in bacteria (Yin et al., 2002), degree of deacetylation of chitin (Prochazkova et al., 1999), degree of cross-linking in collagen/ chitosan scaffolds (Yan et al., 2010).

2.8.6 SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) has been applied in different sectors for polypeptides separation and molecular weight determination (Nur Azira et al., 2012). Among different PAGE techniques, the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been commonly applied due to its simplicity, availability, ease of use, and reproducibility (Chandel & Pahadiya, 2005).

In SDS-PAGE, sodium dodecyl sulphate is used to denature the proteins and a discontinuous polyacrylamide gel is used as a support medium for separating proteins by electrophoresis. The system consists of two gels; a resolving gel to resolve proteins on a molecular weight basis and a stacking gel to concentrate proteins prior to entering the resolving gel. The composition and pH of the stacking gel, resolving gel and electrophoresis buffer are important determinants of finely resolving proteins according to their molecular weights (Chandel & Pahadiya, 2005). All proteins bind the anionic SDS through hydrophobic interaction in the theoretical ratio of 1.4 g SDS per 1g of protein, making the charge-to-mass ratio constant for each protein (Dupont et al., 2018). Therefore, the final separation of proteins depends entirely on the differences in relative molecular weight of polypeptides, where smaller molecules migrate faster than larger molecules in the gel (Chandel & Pahadiya, 2005).

Quantification of proteins using SDS-PAGE is more variable than using urea-PAGE due to the shape and somewhat poor resolution of the bands (Dupont et al., 2018). However, sophisticated analysis software along with densitometer has eliminated the challenges of gel data analysis (Nur Azira et al., 2012).

The uses of SDS-PAGE include identification and quantification of molecules and impurities, estimation of relative molecular weight of proteins and hydrolysates (Zheng et al., 2020), estimation of relative abundance of proteins in a sample (Chandel & Pahadiya, 2005). This technique has been applied in digestibility analysis studies; validation of *in vitro* digestive system (Kopf-Bolanaz et al., 2012), *in vitro* digestibility analysis of β -casein and β -lactoglobulin (Mandalari et al., 2009), study of cheese proteolysis (Žolnere et al., 2019), study of caseins and whey proteins digestibility (Inglingstad et al., 2010).

Chapter 3 Materials and Methods

3.1 Introduction

The main objective of this study was to identify the potential of mung bean protein isolate to partially replace milk for the production of paneer. The preliminary step of the study was to identify the suitable plant protein isolate and the maximum ratio it can replace cow milk for the development of hybrid paneer with satisfactory quality.

The effect of mung bean protein addition on the various physical characteristics of the paneer samples such as colour, rheology and textural properties were determined. The microstructure was also determined using CLSM.

In vitro digestion of the paneer samples was also performed to understand the effect of addition of globular protein (mung bean) on the protein digestibility of the paneer. Ninhydrin assay and SDS-PAGE was done for samples at 0, 10, 30 and 60 min for gastric phase and 70, 120, and 180 min at intestinal phase to study protein hydrolysis and release of free amino N. Microstructure of the samples at 0, 60 and 180 min of digestion was also determined using confocal microscopy.

3.2 Materials

Standard milk having a protein content of 3.4 % was purchased from a local supermarket (PaknSave) in Palmerston North and was stored under refrigerated conditions. The composition of milk according to the packaging label is given in Table 11. Citric acid (Hensells) for the experiment was purchased from a local supermarket. Mung bean protein isolate (Harbin Hada Starch Co., Ltd) used for the study was purchased from Davis trading company, Palmerston North. The composition of the MBPI according to the packaging label is given in Table 12.

Table 11: Composition of milk used for the experiment

Nutritional information		
Composition	Avg. value per serving (250 mL)	Avg. value per (100 mL)
Energy	658 kJ	262 kJ
Protein	8.3 g	3.3 g
Fat, total	8.5 g	3.4 g
saturated	5.6 g	2.3 g
Carbohydrate	11.9 g	4.8 g
Sodium	100 mg	40 mg
Calcium	292 mg	117 mg

Table 12: Composition of mung bean protein isolate

Nutritional information	
Composition	Avg. values per 100 g
Energy	1574 kJ
Protein	70.8 g
Fat	0.6 g
Carbohydrate	19.3 g

The enzymes required for the *in vitro* digestion was obtained from Sigma-Aldrich Pty Ltd (USA). Milli-Q water was used for the preparation of reagents and for the experiments. All the chemicals and reagents used for this study were of analytical grade.

3.3 Methods

3.3.1 Preliminary study

The first phase of this study involved identifying the potential plant protein isolate from soy, pea, hemp, mung bean and plant milks like oat, rice, cashew, and soymilk for partially replacing with cow milk for the manufacturing of hybrid paneer. The protein was selected considering the factors of cost, availability, and its ability to form curd when acidified with citric acid. Different ratios of cow milk to plant protein (100:0, 90:10, 80:20, 70:30, 50:50, 30:70, and 100:0) were looked into identify its potential to be partially replaced with cow milk for the manufacturing of paneer (Appendix A).

3.3.2 Preparation of cow milk paneer

The milk was heated to a temperature of 90°C and was allowed to cool down to 80°C. Citric acid solution (1 %) was heated to 70°C and was added to the milk with continuous stirring till

clear whey separated out. The formed curds were rested for 5 min making sure that the temperature did not fall below 63°C. The curds were then drained and separated using a muslin cloth and were pressed using a cheese press by applying a weight of 5 kg for 20 min. The pressed curds were then immersed in chilled water at 4°C for 2 hr. The paneer was removed from the chilled water and was allowed to drain off the excess water. The paneer was then wrapped using film wrap and was stored at refrigerated conditions (4°C). The flow chart describing the manufacturing of paneer is given in Figure 11 and pictures are given in Appendix B.

3.3.3 Preparation of mung bean protein suspension

Mung bean protein suspension was prepared by adding MBPI to water (3.3 % protein) with continuous mixing using a magnetic stirrer for 30 min at 400 rpm under room temperature.

3.3.4 Preparation of cow milk-mung bean paneer

The cow milk-mung bean paneer was made by adding mung bean protein suspension to cow milk. Rest of the steps were similar to the preparation of cow milk paneer and is according to section 3.3.2 and Figure 11. Pictures related to the manufacturing of cow milk-mung bean paneer are given in Appendix C.

3.3.5 Preparation of mung bean curd

Mung bean curd was prepared by heating mung bean suspension (3.3 % protein) to 90°C then cooling it to 70°C and coagulating using 1 % citric acid solution. This mung bean curd was used in the microstructural image analysis using confocal microscopy to compare with the protein network of CMMBP.

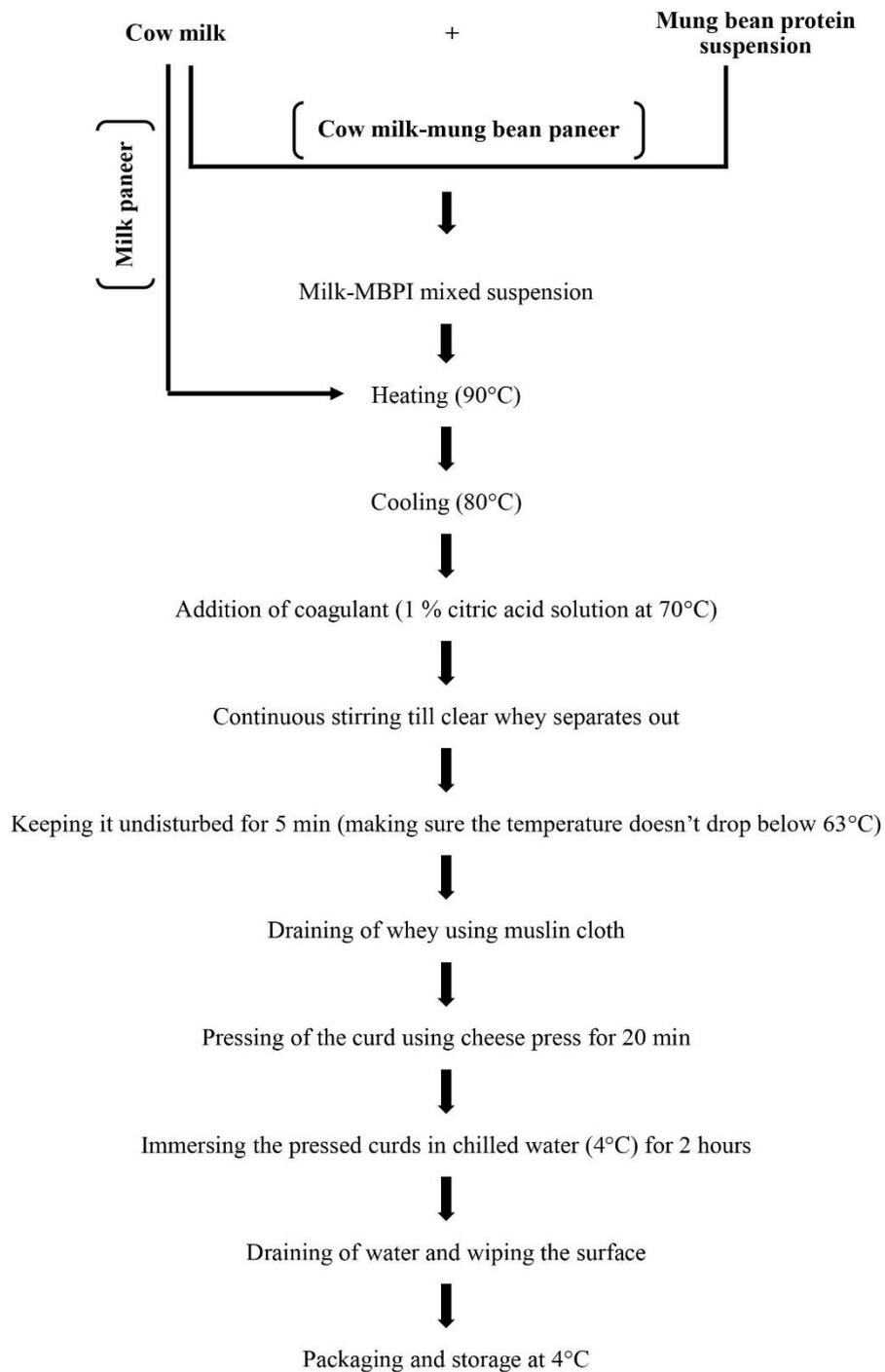


Figure 11: Flow chart describing the manufacture of cow milk paneer and cow milk-mung bean paneer

3.3.6 Composition analysis

The moisture content of the paneer samples was determined using the hot air oven method. A known amount of sample (w_1) was heated at 105°C for 24 hr. Final sample weight was recorded after cooling in a desiccator for 2 hr (w_2). The formula used for the calculation of moisture content is shown in equation 1.

$$\text{Moisture content (\%)} = \frac{(w_1 - w_2) \times 100}{w_1} \quad (\text{Equation 1})$$

The total protein content of the paneer samples was determined using Kjeldahl method using a nitrogen conversion factor of 6.38. A known amount of sample (C) was digested using 2 kjeltabs (each tablet containing 0.0035g Se and 3.5g K_2SO_4 , Thermo Fisher Scientific Pty Ltd., AU) along with 15 mL of concentrated H_2SO_4 acid and placed on a digester at 420°C until the solution became clear (90 min). The digested samples were then distilled and titrated using 0.1 M HCl. The formula used for the calculation of nitrogen content in the sample is shown in equation 2.

$$\% \text{ Nitrogen} = \frac{(A \times B) \times 14 \times 100}{100 \times C} \quad (\text{Equation 2})$$

where, A = mLs HCl used

B = exact molarity (normality) of HCl

C = weight in g of original sample used

The formula used for the calculation of protein content in the sample using nitrogen to protein conversion factor of 6.38 is given in equation 3.

$$\text{Protein content (\%)} = \% \text{ Nitrogen} \times 6.38 \quad (\text{Equation 3})$$

The fat content of the paneer samples was estimated using Soxhlet extraction method. The paneer samples were freeze-dried (Buchi Lyovapor L-300) to remove the moisture and a known amount of sample (w_3) was filled into a cellulose thimble. Fat in the samples was extracted into a pre-weighed (w_4) round bottom flask for 6 hr using petroleum ether as the solvent. After

extraction, the remaining solvent was evaporated in a rotary evaporator and the round bottom flask was placed in a hot air oven at 105°C for 2 hr to remove any remaining moisture. The weight of the round bottom flask was taken after cooling in a desiccator (w_5). The formula used for the calculation of fat content on a dry weight basis is shown in equation 4.

$$\text{Fat content (\%)} = \frac{(w_5 - w_4) \times 100}{w_3} \quad (\text{Equation 4})$$

All the experiments were conducted in triplicate.

3.3.7 Colour analysis

The colour profile of the paneer samples was analysed using Minolta Chroma meter CR 400. The L^* (indicates lightness range between 0 and 100), a^* (Positive a^* value = redness and negative a^* value = greenness) and b^* (positive b^* value = yellowness and negative b^* value = blueness) parameters were determined according to CIELAB colour space. The equipment was calibrated using a white tile ($Y = 86.6$, $x = 0.3162$, $y = 0.3232$) as a standard. The reported value is an average of measurements taken for 2 samples of the same paneer (five measurements per sample).

3.3.8 Small amplitude rheology

Small amplitude oscillatory measurement was performed on paneer samples using a controlled stress rheometer (Anton paar, MCR302, Germany), according to the method given by Hosseini-Parvar et al. (2015). Two different paneer samples were analysed for various rheological properties using a rheometer attached with a 25 mm diameter parallel plate geometry and a gap size of 2 mm. A cylindrical piece of the sample (2 mm) was cut carefully using a mould and wire cutter and was stored in an airtight plastic bag to prevent dehydration. A thin layer of mineral oil was applied around the sample to prevent evaporation. The sample was placed on the surface of the lower plate and the upper plate was moved closer to the sample till it reached the 2 mm gap. Excess sample was removed using a razor. The sample was rested on the rheometer for 10 min to minimise the stress caused during sample handling. A strain sweep (0.01 – 100 %) was performed at 20°C and a frequency of 1 Hz was selected for determining the viscoelastic region of the paneer samples. A Frequency sweep test was performed at 20°C with a strain amplitude of 0.5 % and the frequency ranged from 0.1 to 10 Hz. A temperature sweep was performed at a constant frequency of 0.1 Hz and a constant strain amplitude of 0.5 %. The temperature ranged from 20°C to 80°C at 5°C/min using a Peltier heating system. The

storage modulus (G'), loss modulus (G'') and loss tangent ($\tan \delta$) were determined. $\tan \delta$ is the ratio between loss modulus (G'') and storage modulus (G'). The temperature where $G' = G''$ ($\tan \delta = 1$) is known as the transition temperature and is an indicator of product meltability. All the measurements were taken in triplicate.

3.3.9 Confocal microscopy

The microstructure of CMP and CMMBP was examined using CLSM (Leica SP5 DM6000B scanning confocal microscope). Thin slices of paneer were cut from the middle portion of the freshly cut paneer. These slices were fixed onto a glass slide and were stained using Nile red fluorescent dye for labelling the fat phase and fast green FCF for staining the protein phase. A cover slip was kept on the top of the cheese slide for 30 min, to allow the dye to diffuse through the sample. Excess amount of dye was absorbed using kimwipes (KIMTECH). The stained samples were kept under a light microscope under $63 \times$ oil immersion objective lens and the emission filters were set at 488 nm for Nile red and 633 nm for fast green. The CLSM micrographs were analysed using Image J software.

In a similar manner, mung bean curds were placed on the glass slide, stained, and looked through a confocal microscope as mentioned above.

3.3.10 Texture profile analysis

Texture analyser (TA.XT. Plus Texture analyser, UK) with a load cell capacity of 5 kg was used for determining the texture profile analysis of both CMP and CMMBP. The experiment was performed using a double compression test which generated a force-time graph (Figure 10), that was used to calculate the hardness, springiness, cohesiveness, and chewiness of the samples. The paneer samples were cut into cubes of $10 \times 10 \times 10 \text{ mm}^3$. A cylindrical probe was used to compress the sample up to 70 % of its original length, and the pre-test speed, test speed and post-test speed of the probe was set to 0.5 mm/s, 1 mm/s and 5 mm/s, respectively. The texture parameters were calculated according to (Bourne, 1978). The reported data is an average of 5 measurements for each type of paneer.

Hardness (N) = Maximum force of the first compression

Cohesiveness = Area under 1st compression (Area 2) / Area under 2nd compression (Area 1)

Springiness = Length 1 / Length 2

Chewiness (N) = Hardness \times Cohesiveness \times Springiness

3.3.11 *In vitro* digestion

The *in vitro* digestion of the paneer samples was performed according to (Minekus et al., 2014) with some modifications. *In vitro* digestion experiments were done in triplicate for both CMP and CMMBP. Two separate jacketed glass reactors were used for performing the *in vitro* digestion experiments; first reactor for gastric phase and the second reactor for intestinal phase. These reactors were attached to a circulatory water bath at $37 \pm 1^\circ\text{C}$ (Figure 12) to maintain the human body temperature. 8 grams of paneer sample was crushed using a mortar and pestle for 1 min for mimicking the chewing process of the food and was transferred into the two jacketed glass reactors.

The samples were first incubated with simulated salivary fluid having 1.25×10^{-6} katal/mL of α -amylase (10025, Sigma Aldrich, Saint Louis, MO, USA) and the pH was maintained at 7.0 ± 0.1 for 2 min. This 2 min period represents the oral phase of digestion. It was followed by the addition of stimulated gastric fluid containing 1.33×10^{-7} katal/mL protein of sample pepsin (P7000, Sigma Aldrich, Saint Louis, MO, USA) and the pH was adjusted to 3.0 ± 0.1 . This marks the beginning of the gastric phase. 6 - 7 glass beads of 3 - 5mm diameter were added to mimic the maceration of the paneer in the gastro small intestinal tract. Samples were collected at 0, 10, 30 and 60 min of the gastric phase.

Samples of the intestinal digestion phase were obtained from the second reactor where after 60 min of gastric digestion, the digest was added with stimulant intestinal fluid and pancreatin (P1750, Sigma Aldrich, Saint Louis, USA) at 1:100 pancreatin to milk protein ratio. The pH during the intestinal phase was maintained at 7 ± 0.1 with continuous mixing. The samples during the intestinal phase were collected at 70, 120 and 180 min. 12 μL of Pepstatin A (ab141416 Abcam, UK) in methanol (0.5 mg/mL) was quickly added to every 1 mL gastric digesta taken and 0.45 mL of SIGMAFAST protease inhibitor cocktail solution (S8820, Sigma Aldrich, USA) (1 tablet in 50 mL Milli-Q water) was mixed with every 1 mL of the small intestinal digesta to inactivate the digestive enzymes present in the digesta.

The mixture of the digesta and enzyme inhibitor was dispersed for 30 sec using a handheld disperser (5 mm diameter) (T 10 basic ULTRA-TURRAX Homogenizer, IKA, Germany) at a setting of 3 in an ice bath. The digesta were then stored at 20°C for further analysis.

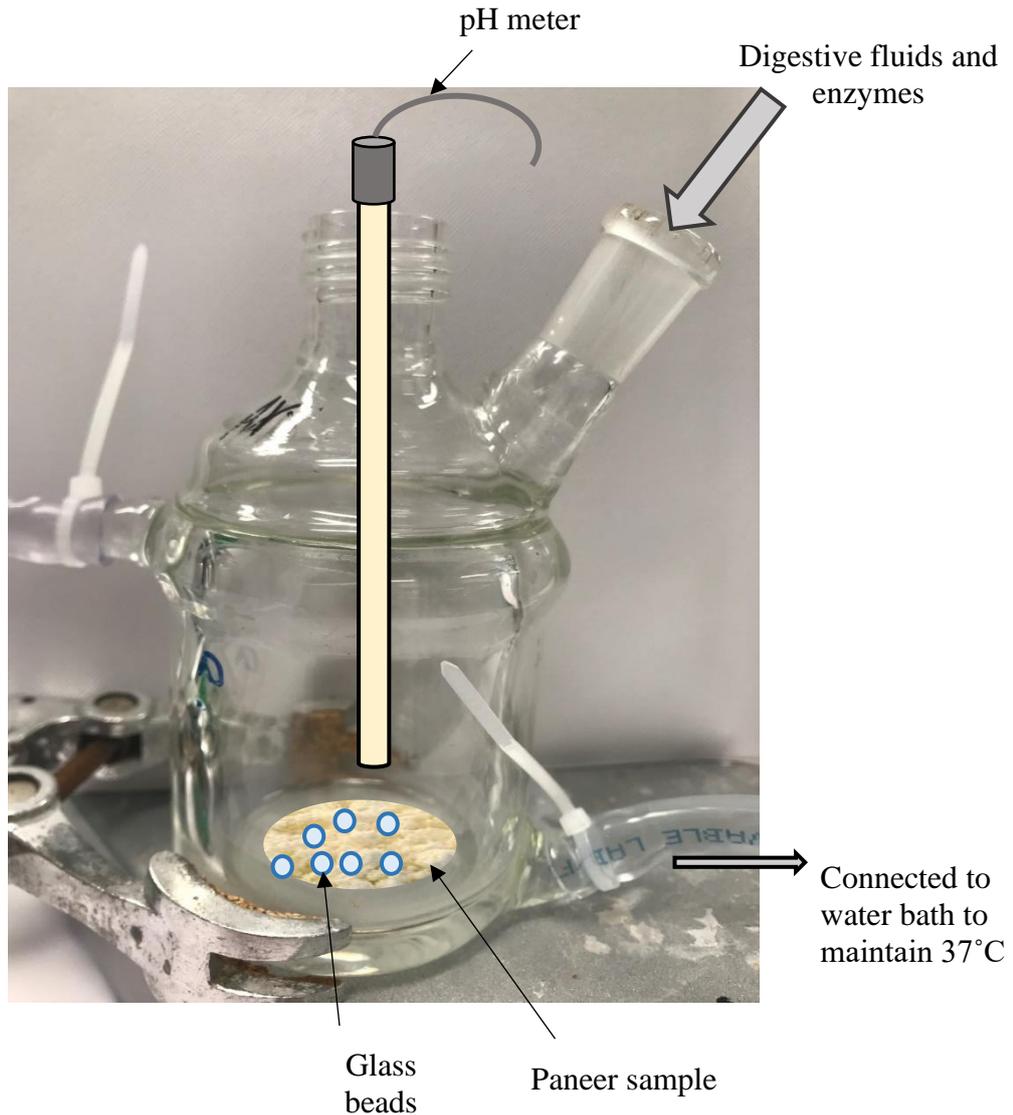


Figure 12: Jacketed glass reactor attached to a circulatory water bath

3.3.12 Ninhydrin reactive amino N

The frozen digested samples from *in vitro* digestion were thawed at room temperature and centrifuged at 14,000 rpm for 7 min (Eppendorf Minispin plus). The supernatant was filtered using a 0.45 μm PVDF syringe filter (Millex). Ninhydrin reactive amino N (%) in the filtered supernatant was determined according to the method described by (Moore, 1968) using ninhydrin reagent (N7285, Sigma Aldrich Pty Ltd, USA).

Table 13: Quantity of samples taken for the ninhydrin assay at different time intervals

Time	0 min	10 min	30 min	60 min	70 min	120 min	180 min
Quantity	15 μ L	20 μ L	8 μ L				

Quantities of samples were taken into Kimax tubes as mentioned in Table 13 and mixed with pH adjusted Milli-Q water; pH of 3 ± 1 for gastric phase samples and pH 7 for intestinal phase samples and made to 1 mL. 0.5 mL of 2 % ninhydrin reagent was added to each Kimax tube. The tubes were then heated at 100°C for 10 min, cooled to room temperature and 2.5 mL of 95 % ethanol was added.

The ninhydrin reactive amino nitrogen concentrations of the samples were determined using a standard curve as follows. A standard series was prepared (0, 0.0125, 0.025, 0.0375 and 0.05 μ mol/mL) according to Table 14 by adding different volumes of a stock solution of 50 μ M glycine in 0.05 % glacial acetic acid into Kimax tubes and made to 1 mL by adding Milli-Q water. To each Kimax tube 0.5 mL of 2 % ninhydrin reagent was added and the absorbance values of the standard series were obtained at 570 nm and the standard curve was plotted as the absorbance (y-axis) against glycine concentration (x-axis). The absorbance values of the sample digesta were obtained at 570 nm and the respective ninhydrin reactive amino nitrogen concentrations were determined using the equation from the standard curve. The absorbance of the samples and standards were obtained using a Vis-spectrophotometer (Helios Epsilon, Thermo Fisher Scientific Pty Ltd, U.K). The absorbance values between 0.2 to 0.8 were accepted.

Table 14: The preparation protocol of the glycine standard series

Standard	1	2	3	4	5
Concentration (μ mol/mL)	0.0000	0.0125	0.0250	0.0375	0.0500
50 μ M of glycine standard (mL)	0.00	0.250	0.500	0.750	1
Milli-Q water (mL)	1	0.750	0.500	0.250	0.00

3.3.13 Confocal microscopy of the digested cheese samples

The digested paneer samples (0 min, 60 min, and 180 min) were stained using Nile red for the fat phase and Fast Green FCF for the protein phase. The samples were rested for 10 min and were examined using a scanning confocal microscope (Leica SP5 DM600B) under a 63× oil immersion objective lens. An excitation wavelength of 480 nm was selected for Nile red, and 633 nm was selected for Fast green FCF.

3.3.14 SDS-PAGE

Gel electrophoresis is a suitable method that helps to understand the breakdown of proteins during the initial stage of digestion to peptides greater than 3.5 kD (Mills et al., 2013). It is a commonly used technique for understanding the digestion rate of individual protein components (Nguyen, Bhandari, Cichero, & Prakash, 2018).

The hydrolysis of proteins in paneer during digestion was examined using reduced-Tricine-SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method performed by Chian et al. (2019). 200 µL of the digested sample was mixed with 100 µL of tricine buffer containing 2 % mercaptoethanol and was submerged in a boiling water bath for 5 min. 25 µL of the digested sample was loaded onto gel (16.5 % gradient Tricine gels, Criterion Precast gels Bio-Rad Laboratories) at a protein concentration of 2 mg/mL along with 10 µL of protein standard (Precision Plus Protein™ All Blue Prestained Protein Standard) as reference. Gels were run using 1×Tricine running buffer (Bio-Rad Laboratories Pty. Ltd, New Zealand) in Criterion cell (Bio-Rad Laboratories Pty. Ltd, New Zealand) at a constant voltage of 125 V, for 2 hr. The gels were then fixed using a fixing solution (40 % methanol + 10 % glacial acetic acid + 50 % Milli-Q water) for about 30 min and were stained using Bio-safe™ Coomassie blue stain (Bio-Rad Laboratories Pty. Ltd, New Zealand) for 1 hour. The gels were then washed and stored in Milli-Q water at 4°C. The gel images were then scanned using a gel scanning densitometer (Molecular imager Gel doc XR, Bio-Rad laboratories Pty. Ltd, New Zealand).

3.4 Statistical data analysis

Microsoft Excel (Microsoft Corporation, U.S.A.) was used for calculating mean ± SD (standard deviation) of all the obtained values and to graphically represent the results. The means were compared to determine the significant difference between the paneer samples by one-way analysis of variance (ANOVA) at $P < 0.05$ significance level using Minitab 19 software (Mintab Ltd., Coventry, U.K.).

Chapter 4 Results and Discussion

This chapter describes and discusses the results obtained for the various analytical techniques (described in Chapter 3) performed on the paneer samples. Due to the lack of articles on paneer incorporated with plant protein isolates, literature available on cheese was mainly used to discuss the results.

4.1 Physicochemical properties and microstructure of paneer

4.1.1 Composition of paneer

Table 15 summarises the moisture, protein, and fat composition of CMP and CMMBP. Moisture content of CMMBP (54.04 %) was significantly higher ($P < 0.05$) than that of CMP (52.15 %). Similarly, the protein content of CMMBP (22.27 %) was significantly higher ($P < 0.05$) than that of CMP (19.2 %). In contrast, CMMBP showed a significantly lower ($P < 0.05$) fat content of 17.61 % compared to 20.84 % fat of CMP which can be explained due to the partial replacement of milk with mung bean protein isolate.

These results are in accordance with the moisture and protein contents of low-fat paneer incorporated with soy protein isolate by Kumar et al. (2011), where moisture and protein contents also showed an increasing trend with the increase of soy protein isolate. According to Kumar et al. (2011), moisture content is related to fat content, in which a higher fat content reduces the moisture-holding capacity of paneer. Similarly, in this study, higher fat content in CMP may have reduced the moisture-holding capacity resulting in low moisture content, and in contrast, low-fat content in CMMBP may have resulted in higher moisture content.

Table 15: Composition of the paneer samples

Composition of paneer samples		
Component	CMP (%)	CMMBP (%)
Moisture	52.15 ± 0.43 ^a	54.04 ± 0.42 ^b
Protein	19.2 ± 0.52 ^a	22.27 ± 1.01 ^b
Fat	20.84 ± 0.50 ^a	17.61 ± 0.56 ^b

Means with different superscript letters are significantly different

An increase in moisture content was also reported by Lu et al. (2010) in cheese developed by partially replacing milk with sesame protein isolate. An increase in moisture content with the increase of sesame protein isolate concentration was also reported. In addition, Rinaldoni et al. (2014) reported higher moisture content in soft cheese incorporated with soy protein concentrate and concluded that it may be due to the hydrophilic nature of soy protein. In addition, the results obtained by Lee and Marshall (1979) showed that soy protein increased the water holding capacity in rennet curd made from soy protein and milk. Mung bean protein has a similar ratio of hydrophilic and hydrophobic amino acids to soy protein (Brishti et al., 2017). Therefore, the higher moisture content in CMMBP might be due to the hydrophilic nature of mung bean protein.

El-Sayed (1997) reported an increase in total nitrogen content in processed cheese incorporated with different plant protein isolates. Rinaldoni et al. (2014) also observed an increase in protein content with the increase in soy protein concentrate.

In conclusion, the results revealed a significant difference between the composition of CMP and CMMBP. The difference may be attributed by the composition of ingredients used for the preparation of the two paneer types. These difference in composition has affected the quality attributes of paneer which has been described in the next sections.

4.1.2 Colour analysis

Colour is an important characteristic of food and has a direct influence on consumer acceptability as well as product appeal (Ramos et al., 2013). Table 16 summarises the colour profile of paneer samples analysed according to CIELAB colour space. It shows a decrease in the L* value for CMMBP (86.37) indicating a slightly darker colour when compared to CMP (89.15) ($P < 0.05$). Similar results were obtained by Kumar et al. (2011) using soy protein isolate as a fat replacer in low-fat paneer. The decrease in the lightness of CMMBP can be due to a decrease in its fat content, as L* value is directly proportional to fat content because of the scattering of light by fat molecules (Kumar et al., 2011).

Table 16: Mean \pm standard deviation for colour parameters of cow milk paneer and cow milk-mung bean paneer

Paneer sample	L*	a*	b*
CMP	89.15 \pm 1.10 ^a	-3.13 \pm 0.14 ^a	17.52 \pm 0.90
CMMBP	86.37 \pm 0.92 ^b	-1.66 \pm 0.07 ^b	18.12 \pm 0.94

Means with different superscript letters are significantly different

The negative a^* value of the samples indicated that CMMBP (-1.66) has a lower greenness in contrast to CMP (-3.13) ($P < 0.05$). Similar a^* value of CMP was obtained by Sant'Ana et al. (2013) for minas fresh cheese made from cow and goat milk (-3.16). Rinaldoni et al. (2014) observed a lower greenness value in soft cheese incorporated with soy protein concentrate and suggested this may be due to the translucent appearance imparted by the removal of fat in low-fat cheese. Similarly, in this study decreased greenness values in CMMBP may be due to a decrease in fat content by the addition of mung bean protein isolate.

The b^* values of the samples indicated that CMMBP (18.12) has a higher yellow colour than CMP (17.52) samples and was not significantly different ($P > 0.05$). Sant'Ana et al. (2013) obtained similar b^* in minas fresh cheese (16.34) made from cow milk and goat milk.

The slightly higher b^* values of CMMBP could be due to the increase in amine compounds which react with amino acids (Millard reaction) to develop dark pigments (Akesowan, 2009). Higher b^* values were obtained by Kumar et al. (2011) in soy protein incorporated paneer and Rinaldoni et al. (2014) in soft cheese developed with soy protein concentrates.

The slightly darker colour of CMMBP compared to CMP was also observed under the naked eye (Figure 13).

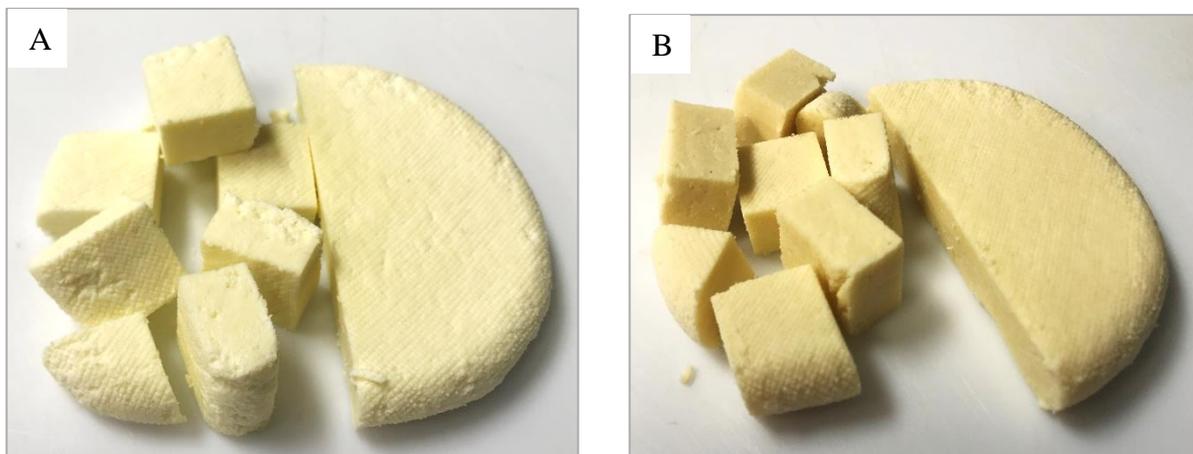


Figure 13: Developed paneer as viewed by the naked eye (A: cow milk paneer; B: cow milk-mung bean paneer)

4.1.3 Small amplitude rheology

The textural and body characteristics of cheese and how they are influenced by processing techniques and composition can be determined by studying their rheological properties (Konstance & Holsinger, 1992). The viscoelastic nature of food can be studied through its small

amplitude oscillatory rheology (Lucey et al., 2003). In this research viscoelastic properties including storage/elastic modulus (G') related to the elastic nature of the material, loss/viscous modulus (G'') related to the viscous behaviour of the material, and loss tangent ($\tan \delta = G''/G'$), which is the ratio of viscous to elastic moduli (Gunasekaran & Ak, 2002) of CMP and CMMBP were determined.

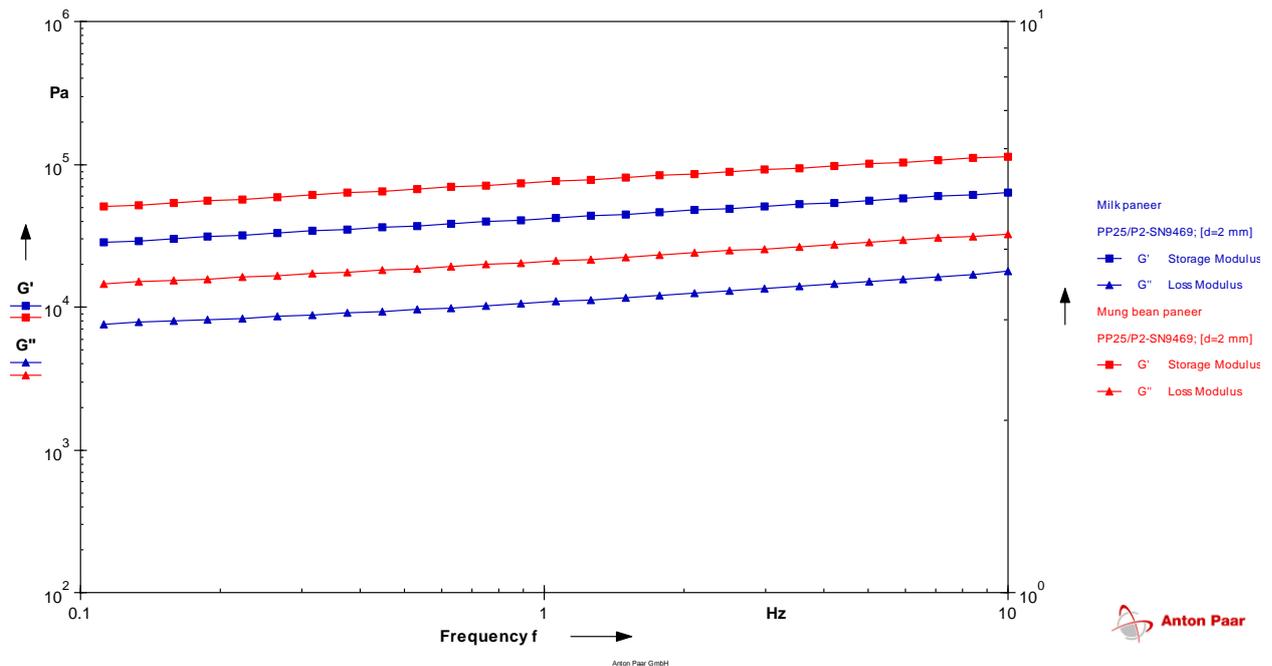


Figure 14: Variation in the storage modulus (G') and loss modulus (G'') in cow milk paneer and cow milk-mung bean paneer with increasing frequency (0.1 - 10) at 20°C

All measurements were done within the viscoelastic region of paneer samples. The measurements within the linear viscoelastic region involve studying the structure of the sample in a non-destructive manner (Kealy, 2006). The amplitude strain sweeps test (frequency 1 Hz) was done to find the linear viscoelastic region for both paneer samples and it lied between 0.01 - 1.5 kPa (Appendix D). A similar viscoelastic range of 0 - 2 kPa was observed for cheddar cheese by San Martín-González et al. (2007).

A frequency sweep test was performed to understand whether partially replacing milk with mung bean protein has an effect on the rheological properties of paneer. A strain amplitude of 0.5 % and a frequency range from 0.1 to 10 Hz was selected for the frequency sweep test. Figure 14 shows the changes in the storage and loss moduli of both CMP and CMMBP as a function of oscillation frequency.

For both CMP and CMMBP, values of G' were significantly higher ($P < 0.05$) than the G'' values. This is an indication of dominant elastic properties. This is a typical behaviour shown by solid viscoelastic materials as reported in several studies on cheese (Juan et al., 2007; Kahyaoglu & Kaya, 2003; Messens et al., 2000; San Martín-González et al., 2007). Kapoor et al. (2021) observed greater G' values in paneer made from cow milk. Similar results were obtained in earlier studies on cheese incorporated with plant proteins such as soft cheese incorporated with soy protein concentrate by Rinaldoni et al. (2014) and whey less feta cheeses substituted with pea protein isolate by Omrani Khiabani et al. (2020).

The significant difference ($P < 0.05$) found between storage (elastic) and loss (viscous) moduli for both CMP and CMMBP samples and the slight variation with the frequency across the whole range of frequencies studied, suggested a strong gel structure (González-Tomás et al., 2008; Rinaldoni et al., 2014).

CMMBP showed higher G' and G'' values when compared to CMP (Figure 14). It may be due to the higher protein content of CMMBP through the incorporation of mung bean protein isolate. According to Farahani et al. (2014), there is an increase in viscoelastic properties with the increase in protein content. Joshi, Muthukumarappan, et al. (2004) found similar results in processed cheese. According to Lobato-Calleros et al. (2000), viscoelastic behaviour of cheese analogues can be modified by changing their composition through incorporating fillers i.e., plant proteins, fats and hydrocolloids that interact with casein protein.

The role of protein in the viscoelastic behaviour of cheese is different from fat and moisture. Viscoelastic properties except for loss tangent increase with higher protein content, which contrasts with the role of moisture and fat in influencing cheese texture (Farahani et al., 2014). This can be a result of increasing number of different intra and intermolecular linkages within the cheese matrix with the increase in protein concentration. This can increase the elastic nature of the cheese resulting in a more solid-like behaviour (Fox et al., 2017). When protein concentration increases, the unfolded protein molecules get closer to each other by attractive forces, thereby the role of proteins in the cheese matrix becomes dominant over that of water and fat (Farahani et al., 2014).

Conversely, Omrani Khiabani et al., (2020) reported a reduction in storage modulus values, lowering the solid-like nature of the product with a higher concentration of plant protein substitution in cheese. The lowering of elastic properties of the cheese may be due to lower

total solids, weaken bonds, and less proteolytic activity of protein components in cheese (Omrani Khiabani et al., 2020).

The current study has not resulted in lower storage modulus values for 30 % mung bean protein isolate incorporated paneer. Therefore, it can be concluded that the replacement of milk with 30 % mung bean protein isolate has no adverse effect on the rheological properties of CMMBP when compared to the control CMP.

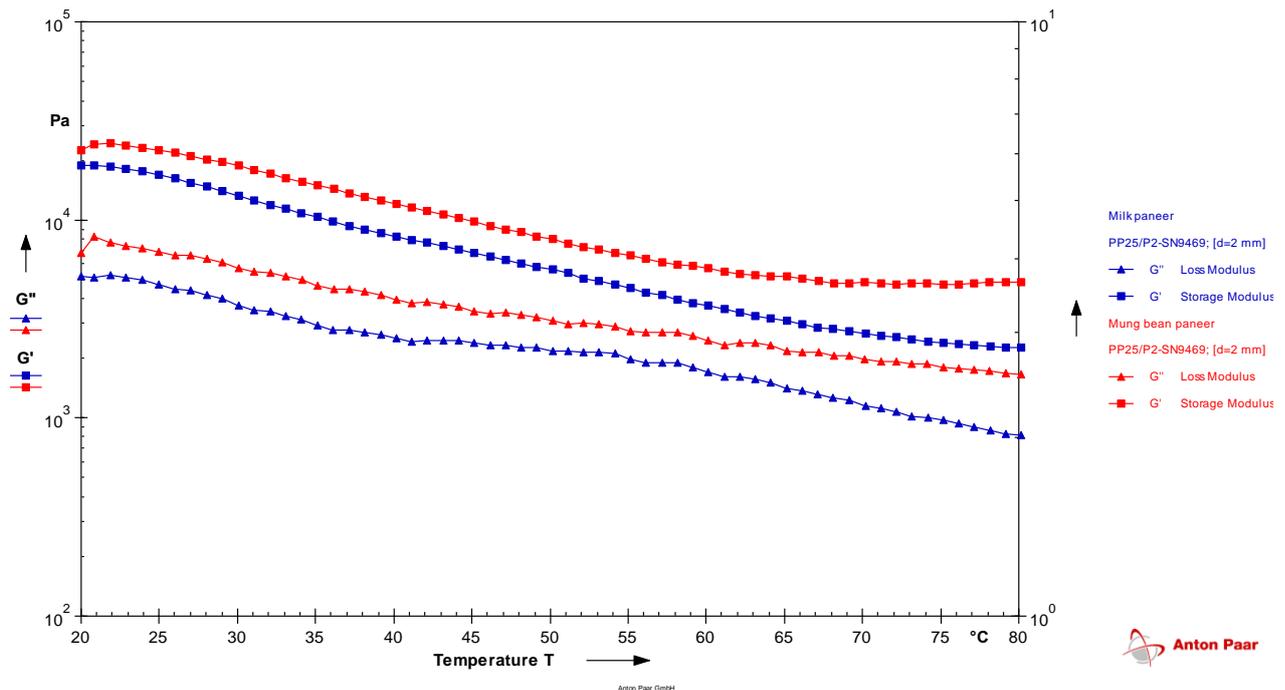


Figure 15: Variation in the storage modulus (G') and loss modulus (G'') in cow milk paneer and cow milk-mung bean paneer with increasing temperature (20 - 80°C) at a frequency of 0.1 Hz

In the case of temperature sweeps (Figure 15), a decrease in the storage modulus (G') and loss modulus (G'') values in both paneer samples were observed with the increase in temperature from 20°C to 80°C. This indicates softening of the paneer with the increase in temperature. Higher G' values compared to G'' values were observed for both paneer samples, indicating a greater elastic nature than the viscous nature of the product throughout the tested temperature range. A temperature sweep indicating crossover temperature for ($G' - G''$) can be identified as a gel-solution transition point (Schenkel et al., 2013). It is evident from Figure 15 that there was no crossover between storage modulus and loss modulus indicating a dominant solid-like nature of the paneer samples.

Tan δ (loss tangent) is the ratio of loss to storage modulus and can be related to the relaxation of bonds within the cheese matrix. It can also be used to indicate the flowability and melting properties of cheese (Mizuno & Lucey, 2005).

Tan δ values less than 1 is an indication of the dominance of elastic nature of the product over its viscous counterpart (Rinaldoni et al., 2014; Van Vliet et al., 1991). According to Figure 16, *tan δ* values of both paneer samples were always less than 1, over the temperature range, indicating a dominant elastic nature of paneer. Lower values of *tan δ* indicate more solid-like properties while higher *tan δ* values indicate liquid-like properties (Lucey et al., 2003). *Tan δ* also indicates a product's structural organisation. Products with highly organised structures show lower *tan δ* values (Létang et al., 1999).

In the case of CMP, *tan δ* values lied between 0.276 and 0.362, while for CMMBP the values lied between 0.354 and 0.284 (Figure 16). Initially, at 20 °C, CMMBP showed a higher *tan δ* value which may be due to its higher moisture content. According to Farahani et al. (2014) and Luyten (1988), *tan δ* increases with increasing moisture content. CMMBP contained higher moisture content than CMP according to moisture analysis data (Table 15). Therefore, the higher *tan δ* values may be due to the higher moisture content in the samples. The behaviour of *tan δ* during latter temperature towards 80°C was opposite to that of initial temperatures, in which CMP recorded higher *tan δ* values than CMMBP. Previous studies have recorded that an increase in fat content can lower the storage and loss modulus values while increasing the *tan δ* values (Farahani et al., 2014). This can be due to the role of fat as a lubricant and filler in the casein matrix, showing a more liquid-like behaviour in cheese texture (Dimitreli & Thomareis, 2008; Subramanian et al., 2006). Due to high temperatures in the latter part, moisture evaporation was possible from both samples. Then, the role of fat may have become more dominant. This may be the reason for higher *tan δ* values recorded for CMP than CMMBP.

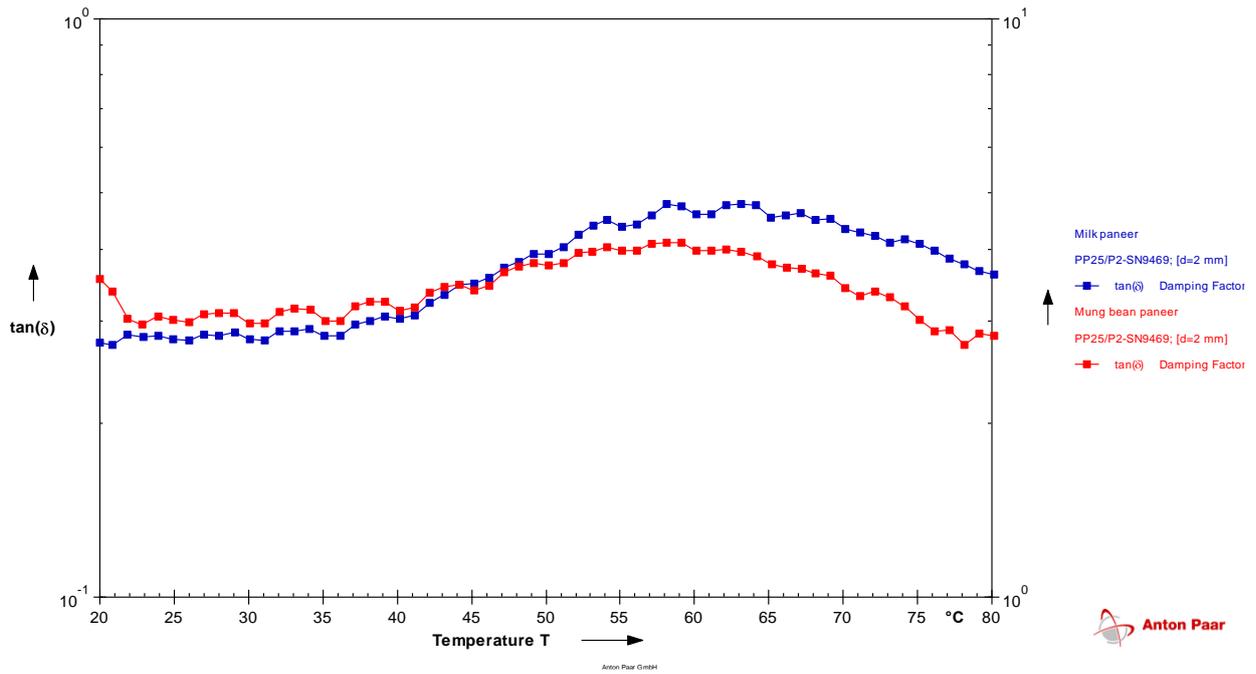


Figure 16: Variation in the $\tan \delta$ value of cow milk paneer and cow milk-mung bean paneer with increasing temperature from 20 - 80°C

4.1.4 Microstructural characteristics

Food microstructure can be defined as the arrangement and interaction of food components depicted at a microscopic level. The cheese microstructure consists of milk fat globules and other components entrapped within the casein matrix (Verboven et al., 2018).

Understanding the cheese microstructure is necessary for manufacturers as well as consumers, as various functional properties and their controlled development depend upon the location and interaction of various components within the cheese matrix. These constituents can be studied through its microstructure, during various processing stages and storage (El-Bakry & Sheehan, 2014).

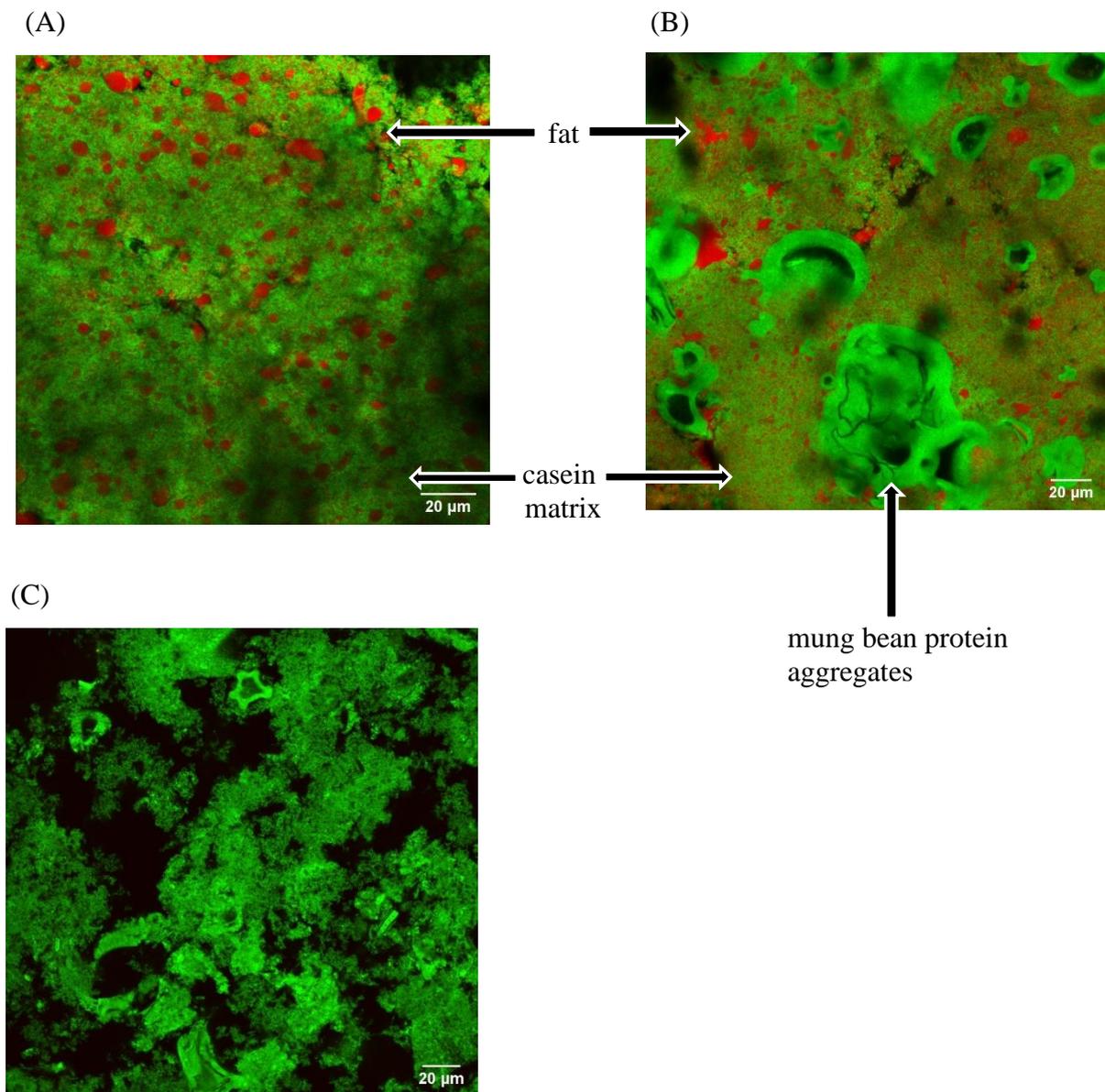


Figure 17: Confocal images of (A) cow milk paneer, (B) cow milk-mung bean paneer (C) mung bean curd

The position of various components within the cheese matrix may be related to their digestibility and release of major nutrients, thereby highlighting the importance of microstructure (Everett & Auty, 2017).

From Figure 17 (A&B), it can be observed that milk proteins formed the primary protein network. In the case of CMP, the fat globules were evenly distributed within the dense protein matrix while in the case of CMMBP, protein aggregates of varying sizes could be observed within the casein matrix along with a few numbers of non-spherical pockets of fat with a higher degree of coalescence. Figure 17 (C) shows the confocal image of the mung bean curd.

The formation of these aggregates in CMMBP can be explained as; first, denaturation during heat treatment due to the unfolding of the tertiary and quaternary structure of both proteins. And then, forming aggregates having high molecular weight due to the exposure of reactive functional groups causing interactions with other particles (Ben-Harb et al., 2018). Similar interactions have been observed by Messian et al. (2015) where, denaturation causes exposure of free thiol-groups, which helps in the formation of disulphide linkages between β -lactoglobulin subunits or/and dissociated legumin subunits. Roesch and Corredig (2005) found that covalent bonds could be created between soy protein subunits and β -lactoglobulin and α -lactalbumin. Ben-Harb et al. (2018) observed that pea/milk mixed emulsion gels during heat/acid treatment showed similar protein aggregates of varying sizes. This aggregate size difference was linked to the highest risk to sample strain, indicating that the formed gels may be brittle in nature (Munialo et al., 2014). Similar bonds must have been created during the denaturation of mung bean protein with milk proteins in this study, which could explain the presence of these large aggregates. When two types of biopolymers, i.e. proteins-polysaccharides, proteins-proteins or polysaccharides-polysaccharides are present in a system during gelation, they may interact with each other before or during gelation and thus affect the final characteristics of the gel network (Ersch et al., 2016).

However, Schmitt et al. (2019) concluded that the addition of plant protein to dairy protein decreases the gel strength when compared to dairy protein alone. This is due to the formation of independent protein networks by dairy as well as plant protein sources that do not interact with each other. They also concluded that micellar casein did not form protein network with plant protein during heating and formed independent networks.

4.1.5 Texture profile analysis

Cheese texture is an important indicator for evaluating the quality and functional properties of cheese as well as for differentiating among various cheese varieties. It also greatly influences customer choices and acceptance (Zheng et al., 2016).

Table 17: Texture profile analysis of the paneer samples

Sample	Hardness	Cohesiveness	Chewiness	Springiness
CMP	17.69 \pm 0.58 ^a	0.34 \pm 0.02 ^a	3.03 \pm 0.11 ^a	0.62 \pm 0.07 ^a
CMMBP	12.81 \pm 0.36 ^b	0.24 \pm 0.01 ^b	0.87 \pm 0.36 ^b	0.34 \pm 0.03 ^b

Each value represents the average of five measurements \pm standard deviation
Means with different superscript letters are significantly different

CMP showed hardness (N), cohesiveness, chewiness, and springiness values of 17.69, 0.34, 3.03, 0.62 (Table 17). Texture profile analysis of various paneer according to previous studies is given in Table 3. Similar hardness, cohesiveness, and springiness values were found by Ahmed and Bajwa (2019) during the manufacture of paneer using citric acid, lemon and amla (Indian gooseberry) juice as the coagulant. Kapoor et al. (2021) found the hardness of paneer to be 10.11 ± 0.29 N. The lower hardness value might be due to the higher fat composition of milk (4 %) used when compared to 3.4 % fat milk used in this experiment. It has been observed that hardness value decreases with an increase in fat content (Uprit & Mishra, 2004).

The textural properties of paneer obtained by Uprit and Mishra (2004) using 3 % fat showed similar textural properties to CMP obtained in this study. Paneer having hardness (16.18 N) values were also produced by Jain and Mhatre (2009) using milk with 3 % fat. However, these values are subjective due to varying composition of milk, type, and concentration of coagulant used and difference in conditions and parameters set for the texture analysis. Therefore, these values can only provide an approximate comparison (Kapoor et al., 2021).

From Table 17 it can be observed that there was a significant decrease ($P < 0.05$) in the textural properties in CMMBP when compared to CMP. The lower hardness of CMMBP might be due to increased moisture content. Researchers like Uprit and Mishra (2004) and Jain and Mhatre (2009) observed similar results on the textural properties of soy paneer made by blending soy milk and skimmed milk in different proportions. They concluded that the decrease in hardness may be due to an increase in moisture content of the paneer and the higher moisture content retained by soy protein (Salinas-Valdés et al., 2015). Similarly, Rinaldoni et al. (2014) reported a decrease in hardness values in soft cheese with the incorporation of soy protein concentrate. Omrani Khiabani et al. (2020) observed a decrease in textural properties with the increase in pea protein isolate in whey-less feta cheese. Both the researchers reported a decrease in hardness values with the increase in plant protein concentration. Mohamed and Morris (1987) also observed a decrease in hardness and increase in water retention in rennet induced coagulum with the addition of soy protein.

The textural parameters are determined by the structural arrangement of the components within the network, which is influenced by the composition and the processing methods employed (Mathare et al., 2009). The microstructure of cheese consists of a continuous protein matrix where other components like water and fat globules are dispersed within the protein network (Bryant et al., 1995). The lower textural properties of cheese may also be due to the presence

of globular plant protein within the casein network which prevents the formation of a compact protein network generally observed in coagulated dairy products thus, weakening the texture (Jain & Mhatre, 2009; Omrani Khiabani et al., 2020). The presence of protein aggregates of varying sizes within the casein network can also be confirmed by the confocal microscopy images in section 4.1.4.

To conclude, high moisture content and the presence of globular nature of mung bean protein present within the paneer matrices may have caused the reduction of textural properties in CMMBP.

Uprit and Mishra (2004) also suggested that hardness values are inversely proportional to fat content. However, in CMMBP despite having lower fat content, it showed lower hardness values which might be due to a much stronger effect of mung bean protein aggregates within the milk protein network.

4.2 *In vitro* digestion of paneer samples

4.2.1 Ninhydrin reactive amino N

Ninhydrin test was performed as a quantitative measurement of free amino N produced during simulated gastro-small intestinal digestion to assess the extent of proteolytic digestion of proteins in paneer.

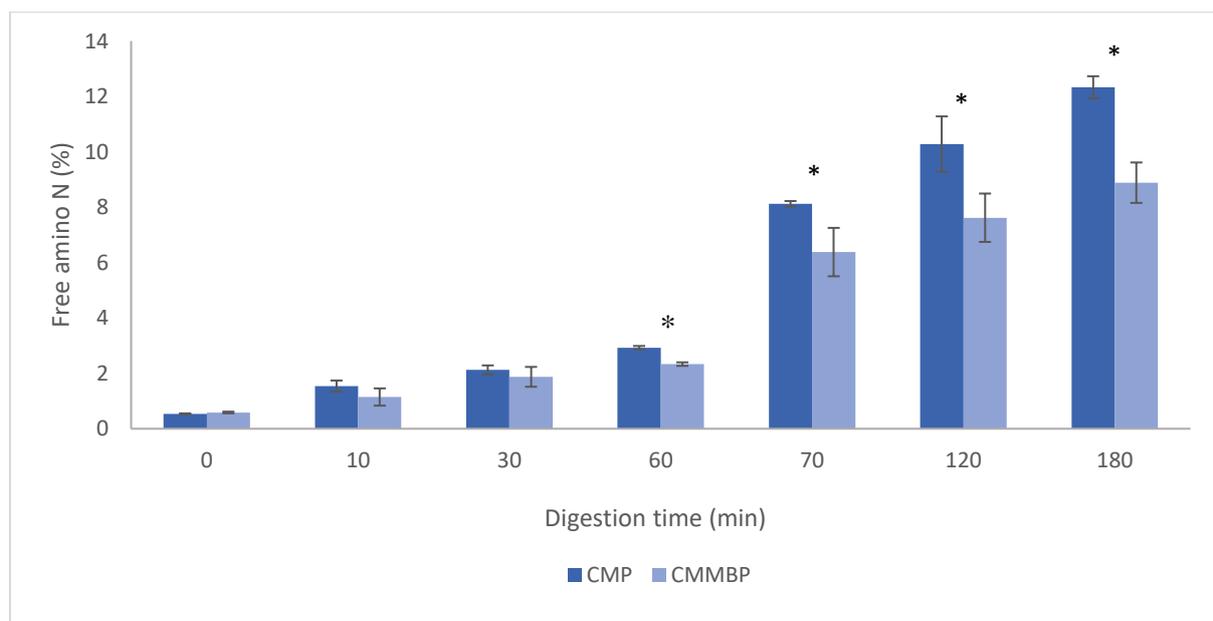


Figure 18: Free amino N released (%) during simulated gastric phase (0 – 60 min) and small intestinal phase (70 – 180 min) in cow milk paneer (CMP) and cow milk and mung bean paneer (CMMBP). Measurements were

made in triplicate and the error bars represent the standard deviations. The bars having a * are significantly different from each other ($P < 0.05$)

There was an increase in the release of free amino N with an increase in digestion time from 0 min to 180 min in both CMP and CMMBP (Figure 18). CMMBP showed a similar release pattern when compared to CMP, but a lower release of free amino N. Hydrolysis of both the paneer samples by pepsin during the gastric phase (0 min - 60 min) was limited and no significant difference was observed between the two samples. Further, with the addition of simulated small intestinal buffer during the small intestinal phase, a rapid increase in the release of free amino N was observed in the two digested paneer samples. However, the release of free amino N remained significantly lower ($P < 0.05$) in CMMBP than CMP. It is understood from this result that the addition of mung bean protein has reduced the free amino N release in the case of CMMBP.

The results are in accordance with the generally accepted fact that animal proteins are better digested when compared to plant proteins (Savoie et al., 2005). Researches on the digestion of matrices like yoghurt and cheese are limited in contrast to purified dairy proteins like whey and casein (Dupont & Tomé, 2020), therefore information is limited to compare the digestibility of paneer in this study with results on similar matrices previously published.

In this study, observations are in accordance with the results obtained by Egger et al. (2019) who compared the static *in vitro* digestibility and dynamic *in vitro* digestibility of milk proteins with *in vivo* results. The results obtained for static *in vitro* digestion of milk proteins showed a very minimal increase in the release of free amino acids (FAA) during the gastric phase compared to small intestinal phase, which might be due to the inactivation of pepsin, by-product inhibition, or pepsin autolysis. In the case of dynamic *in vitro* digestion, a slow increase in the release of free amino N was observed which can be explained by the continuous addition of pepsin, as demanded by the dynamic protocol. Even though there was a gradual increase, the amount released was very low compared to small intestinal phase. The results of the current study also agreed with the experiments conducted by Boutrou et al. (2013) who observed low levels of FAA during the gastric phase compared to small intestinal phase from *in vivo* studies in humans. Similarly, low levels of FAA were observed in pig assay during the gastric phase by Egger et al. (2017). Egger et al. (2016) also identified low levels of FFA release in the gastric phase during *in vitro* digestion of skim milk proteins.

A clear difference can be observed in the release of FAA at the beginning of the small intestinal phase with the addition of small intestinal enzymes. The results were similar to the one observed by Egger et al. (2019); Egger et al. (2016) and Santos-Hernández et al. (2020). Similar results were observed by Lorieau et al. (2018) where the action of pepsin on mozzarella cheese showed limited proteolysis while the addition of small intestinal proteolytic enzymes like trypsin and chymotrypsin showed an increase in the release of α -amino acid groups. The results obtained by Lamothe et al. (2017) also showed an increase in proteolysis with digestion time. Protein hydrolysis has been also reported to be dependent on the type of matrix, the results showed that the rate of hydrolysis decreases with the solid nature of the matrices i.e. liquid matrix (milk) > semi-solid matrices (yoghurt) > solid matrices (cheese) (Lamothe et al., 2017). Legume proteins are generally associated with low digestibility due to the presence of anti-nutritional factors, the structural arrangement of proteins, and complex formation due to the interaction of proteins with other seed components. Their unique compact structure and 3-dimensional stability due to carbohydrate moiety can also lead to cause low digestibility (Tang et al., 2009).

Another reason for low digestibility could be due to the presence of β -sheet conformation in legume proteins which is similar to β -Lactoglobulin present in bovine milk. The presence of this conformation is associated with resistance to denaturation during gastrointestinal digestion (Carbonaro et al., 2015). The digestibility of proteins greatly depends on the extent of denaturation. It is understood that proteolysis can only occur if the protease active site can bind to the specific stereochemistry of the protein to be digested (Lorieau et al., 2018).

Savoie et al. (2005) studied the peptide release kinetics of casein, cod protein, soy protein, and gluten and found that the peptides which were more resistant to digestion were rich in amino acids like proline and glutamic acid. Mung bean protein is rich in these two amino acids and it can be the reason for its low digestibility.

4.2.2 Confocal microstructure of paneer digesta

During digestion the food is partially disintegrated allowing the trapped nutrients to mix with the digestive fluids (Kong & Singh, 2008). The effect of paneer breakdown and protein release was studied during its gastric, and intestinal phase with the addition of enzymes like pepsin, pancreatin and bile salts.

At 0 min, both paneer samples showed an initial breakdown in their protein matrix. Mixing of the paneer samples with gastric enzymes and electrolytes causes phase separation and physical

instabilities of emulsions affecting protein digestion. It also results in release of fat with the partial breakdown of protein structure. CMMBP showed higher amounts of fat droplets being released from the matrix (Figure 19). In contrast, the fat release was lower in CMP. This could be explained due to the structure and material characteristics of both the paneer samples. The lower hardness and cohesiveness values of CMMBP must have led to its faster disintegration and fat release. The bigger size of fat droplets as indicated by arrows (Figure 19 A & D) can be explained due to fat globules retaining its native spherical shape due to re-emulsification by protein when released from the protein matrix, similar observations were reported by Žolnere et al. (2019). Results obtained by Guo et al. (2014), also showed that soft whey protein emulsion gels had a faster disintegration rate and fat droplets release when compared to hard whey protein emulsion gels using *in vitro* gastric digestion.

Significant breakdown of the protein matrix was observed after 60 min of gastric digestion in both the paneer samples (Figure 19 B & E). After 180 min of digestion, the protein-matrix of the CMP was completely disintegrated with few small protein structures remaining when compared to CMMBP which showed a huge number of larger protein aggregates. The disintegration of paneer matrix was higher during intestinal phase compared to gastric phase. In the case of both paneer samples, reduction in the size of fat globules was observed during intestinal phase. The results obtained were similar to the ones obtained by Žolnere et al. (2019). The increase in the release of α -amino N in both paneer samples during the small intestinal phase, which is evident from the results of ninhydrin analysis in section 4.2.1 can be due to the near-complete disintegration of the protein matrix as shown in Figure 19 (C&F).

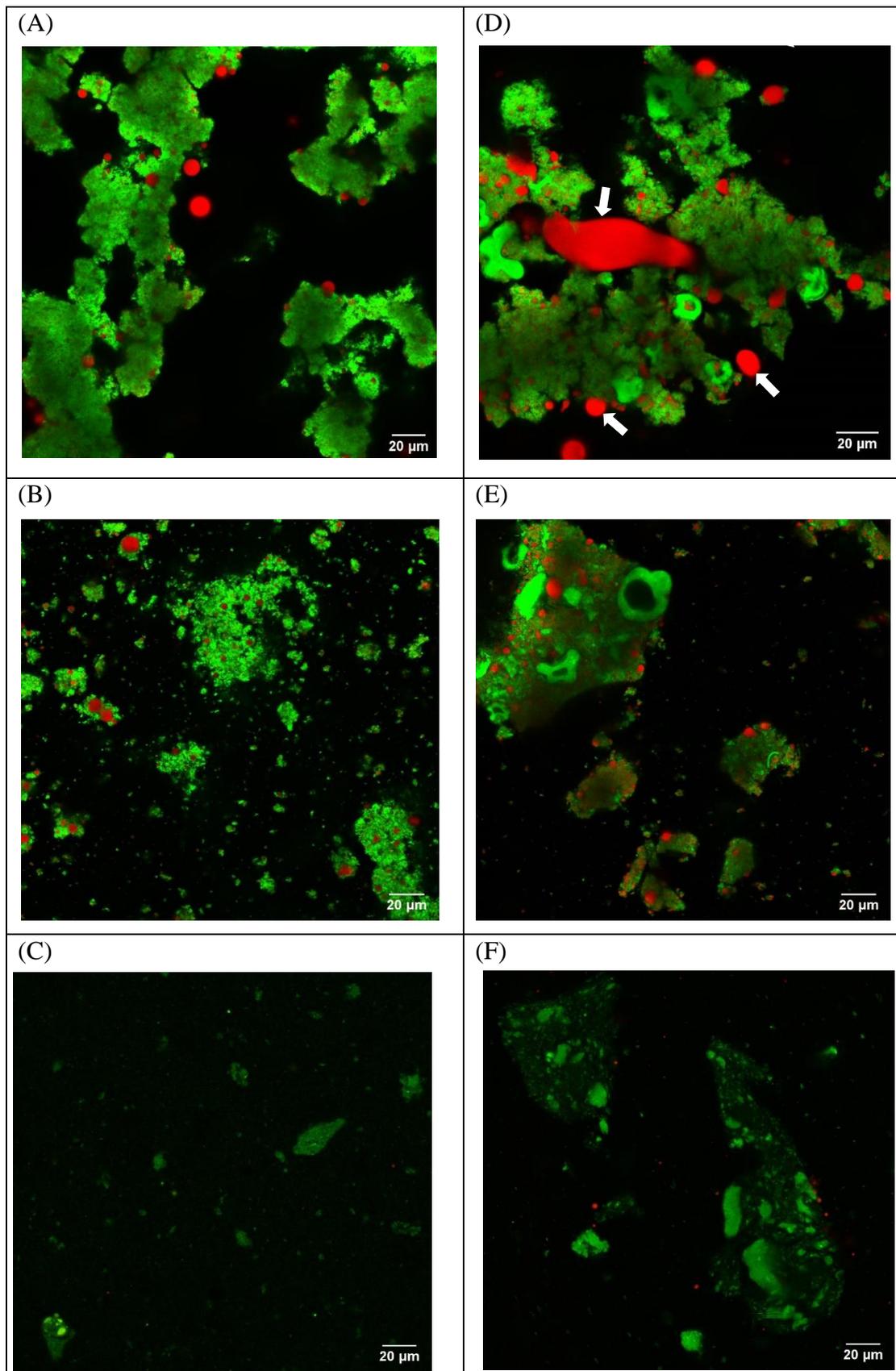


Figure 19: Confocal microscopy images of cow milk paneer (CMP) at 0 min (A), 60 min (B), 180 min (C) and cow milk-mung bean paneer (CMMBP) at 0 min (D), 60 min (E) and 180 min (F). Fats are shown in red, and proteins are shown in green

4.2.3 SDS-PAGE

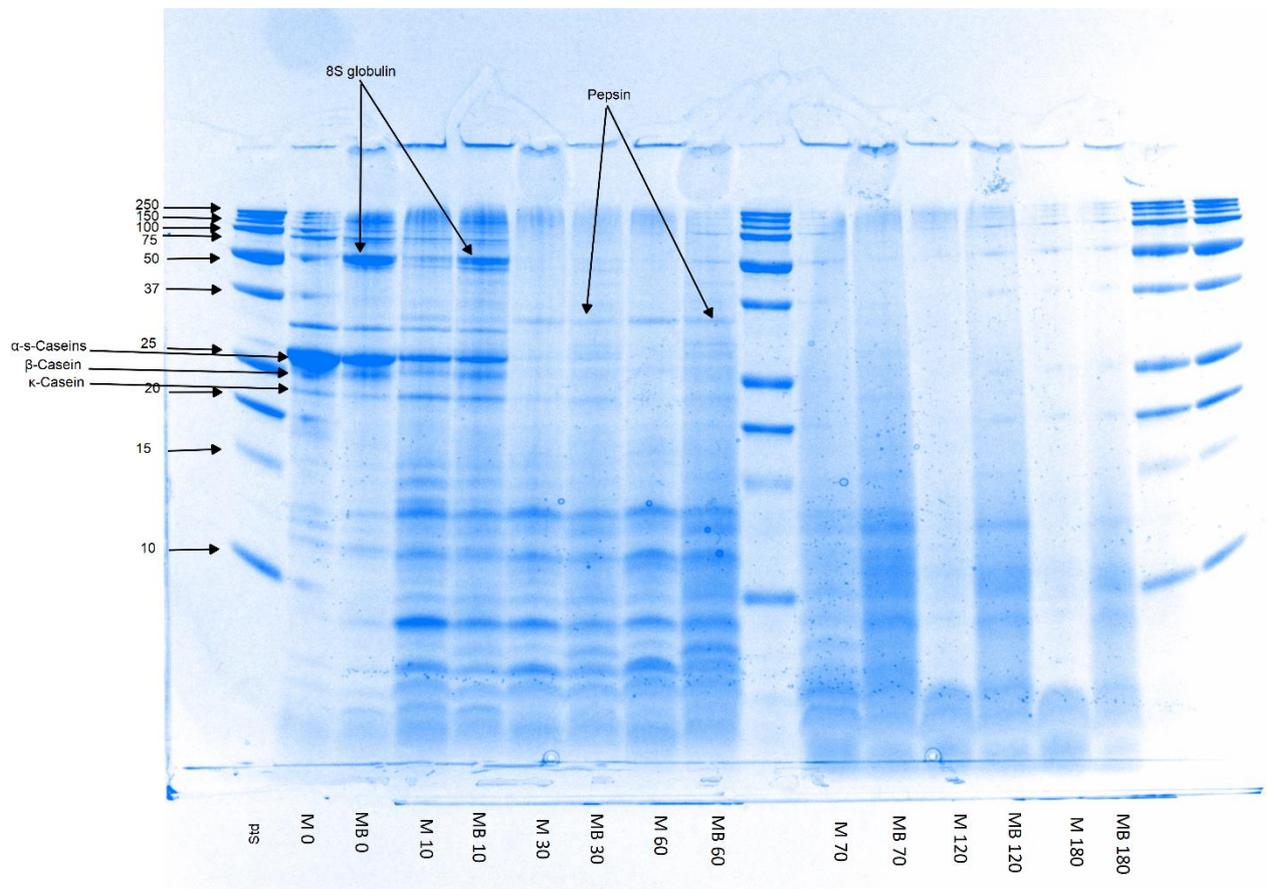


Figure 20: SDS-PAGE gels of hydrolysed paneer samples during gastric (0, 10, 30, 60 min) and small- intestinal (70, 120, 180 min) digestion phases for cow milk paneer and cow milk-mung bean paneer. M = cow milk paneer and MB= cow milk-mung bean paneer

The hydrolysis of protein during *in vitro* gastro-small intestinal digestion of paneer was characterised using SDS-PAGE.

The analysis of CMP and CMMBP samples at gastric (0, 10, 30, 60 min) and intestinal phase (70, 120, 180 min) was compared in Figure 20. With the increase in digestion time, there was an increase in low molecular weight peptides in both the paneer samples, indicating protein hydrolysis.

At 0 min, the bands for casein can be clearly observed in both the paneer samples. After 10 min of gastric digestion, it can be noted that the casein bands have disappeared completely in both the paneer samples. Fang et al. (2016a) observed faster disintegration of casein during the

gastric phase in Camembert cheese. Similarly, results obtained by Parrot et al. (2003) showed that caseins were quickly hydrolysed by the action of pepsin in Emmental cheese. Casein proteins are highly susceptible to proteolysis due to the preference of pepsin enzyme to more loosely structured polypeptides (Dupont & Tomé, 2020).

Whey proteins like α -lactalbumin and β -lactoglobulin particularly β -lactoglobulin are considered to be resistant to proteolysis by pepsin due to its well defined globular intact structure which blocks the enzymes from binding to the potential cleavage sites (Egger et al., 2016; Lorieau et al., 2018; Nguyen et al., 2015; Žolnere et al., 2019). Very low-intensity bands corresponding to α -lactalbumin and β -lactoglobulin were observed in the samples as it is commonly released in whey (Fang et al., 2016b), which is removed during paneer making.

Whey proteins can also form large aggregates with globulins present in mung bean protein through di-sulphide linkages. Minor interactions between whey and soy protein isolate were observed by McCann et al. (2018). During heating, whey proteins can unfold and can form di-sulphide linkages with other proteins as well as soy protein (McCann et al., 2018). The formation of similar aggregates with mung bean protein during digestion can prevent their separation in gels causing low-intensity bands. Therefore, non-reducing SDS-PAGE may be done to identify if there is any aggregate formation between whey proteins and mung bean globulins.

In the case of CMP, after 10 min of small intestinal digestion, the peptides present were completely hydrolysed by the action of enzymes. Fang et al. (2016a) observed that the proteins were rapidly hydrolysed into smaller peptides and amino acids with the addition of intestinal juices and concluded that milk proteins are highly bio-accessible. A similar pattern of protein hydrolysis was observed by Egger et al. (2019) during static *in vitro* digestion of milk proteins. Egger et al. (2016) found similar results while studying the *in vitro* digestion of skim milk powder using INFOGEST protocol.

While, in the case of CMMBP, certain bands were observed around 50 kDa. According to Kudre et al. (2013), the major bands observed for MBPI had molecular weights of 42, 48 and 54 kDa. These bands were hydrolysed into peptides of smaller molecular weights within 10 min of gastric digestion.

During the small intestinal phase, certain bands were observed around 10 -15 kDa which indicates the presence of enzyme-resistant peptides. This resistance to proteolysis in CMMBP might be due to the presence of anti-nutritional factors like trypsin inhibitors, polyphenols, and

phytic acid (El-Moniem, 1999). The presence of trypsin inhibitors can affect the structure of globular protein and can prevent the digestive action of enzymes in the small intestine. Polyphenols present can form complexes with gastric enzymes causing inactivation thus, lowering protein digestibility (Sá et al., 2020).

In the case of CMP, SDS-PAGE results showed that the protein bands disappeared with the increase in digestion time while in the case of CMMBP protein bands were still visible during the small intestinal phase. This can explain the higher amount of free amino N released in CMP during *in vitro* digestion when compared to CMMBP, which is evident from the ninhydrin results.

Cheese texture is known to play a critical role in the rate of cheese disintegration during digestion (Fang et al., 2016a). Foods with lower initial hardness are known to disintegrate faster when compared to foods with higher hardness (Kong & Singh, 2009). In this experiment, CMMBP due to its lower textural properties might have disintegrated faster but showed lower rates of protein hydrolysis, which could be due to the globular nature of mung bean protein and also due to the presence of enzyme resistant peptides.

Chapter 5 Conclusion and Recommendations

Partially replacing dairy proteins with plant proteins can be an optimum solution to tackle the issue of growing protein demand and is a great opportunity to develop novel food products with high nutritional profile in a sustainable manner. This study was an attempt to identify the possibility of developing paneer by partially replacing milk with plant protein isolate. The plant protein isolate of focus of this study was mung bean protein isolate. It was observed that paneer could be successfully developed by partially replacing 30 % of cow milk with mung bean protein isolate.

The results revealed that the paneer made by partially replacing 30 % cow milk with mung bean protein isolate had higher protein and moisture levels compared to cow milk paneer which can be explained due to high moisture retention by mung bean protein. Small amplitude oscillatory tests showed higher G' values than G'' values throughout the frequency range and showed higher G' values in CMMBP compared to CMP, indicating greater elastic properties. Confocal microscopy results showed the presence of protein aggregates of varying sizes within the paneer microstructure with the addition of MBPI, thus preventing the formation of a compact protein network. Texture profile analysis of the paneer samples showed that textural properties like hardness, cohesiveness, springiness, and chewiness values reduced significantly in CMMBP compared to CMP which can be explained due to its high moisture content and the presence of protein aggregates within the paneer microstructure leading to a weak structure.

Furthermore, *in vitro* gastro-small intestinal digestion experiments performed on the samples showed lower protein digestibility of CMMBP. Ninhydrin results showed a lower release of free amino N during the gastric phase than in small intestinal phase in both CMP and CMMBP. A significantly lower release of free amino N was observed in CMMBP compared to CMP during intestinal phase, which was supported by the results obtained by SDS-PAGE which showed the presence of resistant peptides. Overall, the low protein digestibility of CMMBP may be due to the presence of anti-nutritional factors, and complex formation due to the interaction of proteins with other seed components. Confocal microstructure of the paneer digesta also showed lower disintegration in CMMBP after 180 min of digestion.

Further studies need to be done for improving the textural properties of CMMBP. Addition of calcium chloride is known to improve the textural characteristics of paneer and different cheeses due to the formation of protein cross-linkages (Khan & Pal, 2011), hence there is a potential to apply it in CMMBP as well. In addition, different hydrocolloids such as carboxymethyl cellulose and pre-gelatinised starch are known to improve the textural and sensory properties of paneer. The study of these effects on CMMBP will be another opportunity to improve its quality.

Mixing legume proteins with cereal proteins like rice proteins along with dairy proteins can be an interesting opportunity to create dairy product analogues with unique properties. Cereal proteins are rich in sulphur-containing amino acids like cysteine and methionine that can form di-sulphide linkages with other proteins and can create better-structured products. This can also help to create products with complete amino acids composition as legume proteins lack sulphur-containing amino acids while cereal proteins are rich in them (Talbot-Walsh et al., 2018). Legume proteins are generally known for their beany off-taste, addition of seed protein isolate like hemp seed protein can help to eliminate this problem and develop products with an appealing nutty flavour.

Lower digestibility of CMMBP compared to CMP because of addition of mung bean protein isolate was another drawback shown in the results of this study. Various processing techniques such as, soaking, germination, microwave, irradiation, extrusion and fermentation can improve the digestibility of plant proteins (Sá et al., 2020), hence the study of the effect of different processing techniques of plant proteins on the digestibility of paneer will be another interesting future focus to improve the quality of CMMBP.

Future studies can also investigate the microbial study of plant protein incorporated paneer as it is an important aspect to consider in food product development. Further, studies can be expanded to study the sensory aspects of CMMBP as well.

In addition to the research on improving the quality of plant protein incorporated paneer, health benefits of such novel food products associated with the presence of bioactive peptides can also be explored.

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Appendix A: Preliminary tests

For the preparation of the plant protein incorporated paneer several preliminary trials were performed by partially replacing cow milk with several plant protein isolates like, soy, pea, mung bean and hemp protein as well as several plant-based milks like soy, oat, cashew, and rice milk. During the initial trials the idea of mixing plant-based milks with cow milk were discarded due to various factors like high cost, low protein content and less availability.

One of the major objectives of this preliminary study was to identify the maximum ratio that cow milk can be replaced using plant protein for the manufacturing of paneer. To achieve this objective, soy, pea, mung bean and hemp protein isolates were made into suspensions having 3.3 % protein which is similar to bovine milk. Cow milk was mixed with plant protein suspensions in different ratios (100:0, 90:10, 80:20, 70:30, 50:50, 30:70, 0:100) and was made into paneer according to the method described in section 3.3.4. It was observed that all protein isolates were not able to form paneer like product at or below 50:50 ratio. The curds produced were very fine and could not be pressed into paneer. All protein isolates above 70:30 ratio was able to partially replace cow milk to form a paneer like product. Therefore, 70:30 ratio was selected as the most suitable ratio to conduct other experiments.

Another objective of the preliminary study was to identify the most suitable plant protein to make paneer by partially replacing cow milk (Table A).

Cow milk - soy protein paneer has been studied in the past and few researchers have also used soy protein for the manufacturing of paneer. Moreover, due to the sustainability concerns and allergenic nature associated with soybean, soy protein was not considered for this study.

Cow milk - hemp protein paneer showed a darker appearance and grainy taste when consumed due to the presence of black insoluble particles and hence was not selected.

Cow milk - pea protein paneer and cow milk-mung bean paneer performed quite similar to each other in terms of visual appearance, mouthfeel and texture. However, cow milk-mung bean paneer showed higher yield when compared to cow milk - pea protein paneer.

$$\text{Yield} = (\text{quantity of paneer (g)}/\text{quantity of milk used (g)}) \times 100$$

Mung bean protein was selected for this experiment due to its sustainable nature, low allergenicity and gelation ability.

Table A: Comparison of the results of the experiments done to select the most suitable plant protein to partially replace cow milk

Cow milk - plant protein	Observations
mixed paneer (70:30)	
Cow milk - soy protein paneer	<ul style="list-style-type: none"> • Paneer like product could be obtained. • Good texture • Beany flavour • Colour similar to cow milk paneer
Cow milk - hemp protein paneer	<ul style="list-style-type: none"> • Paneer like product could be obtained. • Dark appearance • Presence of black insoluble particles • Brittle on press • Grainy taste
Cow milk - pea protein paneer	<ul style="list-style-type: none"> • Paneer like product could be obtained. • Harder texture and compact nature • Yellowish colour • Crumbly texture
Cow milk-mung bean protein paneer	<ul style="list-style-type: none"> • Paneer like product could be obtained. • Similar colour to cow milk paneer • Held the texture when pressed to some extent • Grainy and crumbly texture

Appendix B: Flow chart showing the preparation of cow milk paneer

Heating milk to 90°C



Addition of citric acid



Coagulation



Cow milk paneer

Pressing

Draining of whey

Appendix C: Flow chart showing the preparation of cow milk-mung bean paneer

Addition of mung bean suspension to cow milk



Heating the mixture to 90°C



Addition of citric acid



Cow milk-mung bean paneer



Pressing

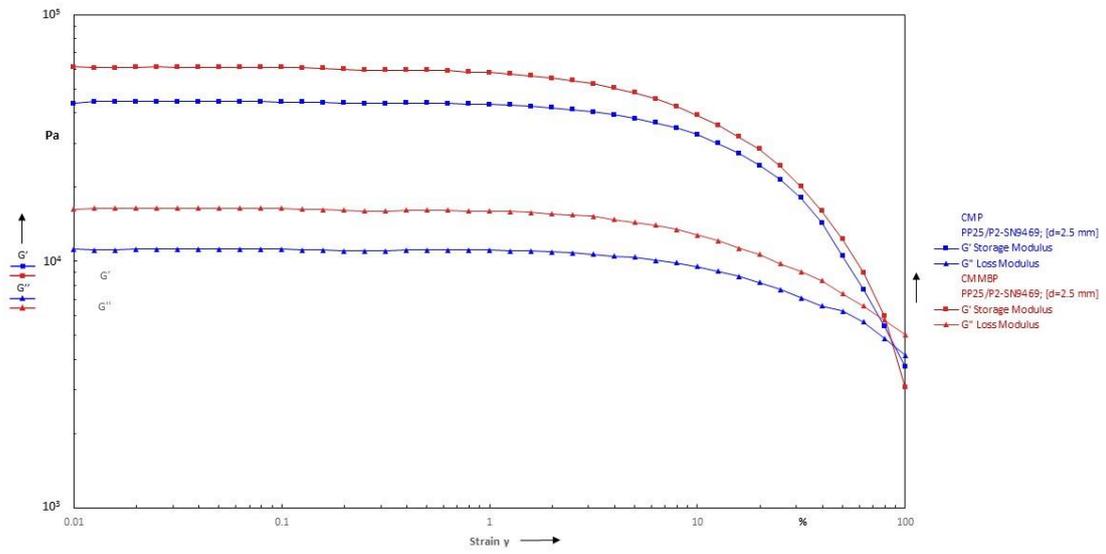


Draining of whey



Coagulation

Appendix D: Amplitude sweep performed on the paneer samples



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Mung bean proteins and peptides: nutritional, functional and bioactive properties

[Zhu Yi-Shen](#)^{1,*}, [Sun Shuai](#)¹ and [Richard FitzGerald](#)²

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